(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(54) Title: MONATIN TABLETOP SWEETENER COMPOSITIONS AND METHODS OF MAKING SAME

(57) Abstract: The present invention relates to novel sweetener compositions comprising monatin and methods for making such
compositions. The present invention also relates to sweetener compositions comprising specific monatin stereoisomers, specific
blends of monatin stereoisomers, and /or monatin produced via a biosynthetic pathway in vivo (e.g., inside cells) or in vitro.

MONATIN TABLETOP SWEETENER COMPOSITIONS AND METHODS OF MAKING SAME

CROSS-REFERENCE TO RELATED APPLICATIONS

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This application claims the benefit of U.S. Provisional Patent Application 60/492,014 filed August 1, 2003, the entire disclosure of which is incorporated herein by reference.

10
FIELD OF INVENTION

The present invention relates to novel sweetener compositions comprising monatin and methods for making such compositions. The present invention also relates to sweetener compositions comprising specific monatin stereoisomers, specific blends of

15   monatin stereoisomers, and/or monatin produced via a biosynthetic pathway *in vivo* (e.g., inside cells) or *in vitro*.

BACKGROUND

20   The use of non-caloric high intensity sweeteners is increasing due to health concerns raised over childhood obesity, type II diabetes, and related illnesses. Thus, a demand exists for sweeteners having a sweetness significantly higher than that in conventional sweeteners, such as granulated sugar (sucrose). Many high intensity sweeteners contain unpleasant off-flavors and/or unexpected and less-than-desirable sweetness

25   profiles. In attempts to overcome these problems, the industry continues to conduct significant research into bitterness inhibitors, off-flavor masking technologies, and sweetener blends to achieve a sweetness profile similar to sucrose.

Monatin (2-hydroxy-2-(indol-3-ylmethyl)-4-aminoglutaric acid) is a naturally-

30   occurring, high intensity sweetener isolated from the plant *Sclerochiton ilicifolius*, found in the Transvaal Region of South Africa. Monatin contains no carbohydrate or sugar, and nearly no calories, unlike sucrose or other nutritive sweeteners at equal sweetness.

SUMMARY

The present invention relates to sweetener compositions comprising monatin (2-hydroxy-2-(indol-3-ylmethyl)-4-aminoglutaric acid—also known as 4-amino-2-hydroxy-2-(1H-indol-3-ylmethyl)-pentanedioic acid, or alternatively, based on an alternate numbering system, 4-hydroxy-4-(3-indolylmethyl) glutamic acid), a compound having the formula:
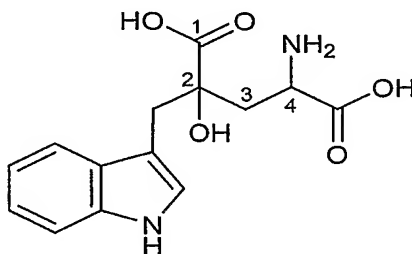
Monatin is a naturally-occurring, high intensity sweetener. Monatin has four stereoisomeric forms: 2R, 4R (the "R,R stereoisomer" or "R,R monatin"), 2S, 4S (the "S,S stereoisomer" or "S,S monatin"), 2R, 4S (the "R,S stereoisomer" or "R,S monatin"), and 2S, 4R (the "S,R stereoisomer" or "S,R monatin"). As used herein, unless stated otherwise, "monatin" refers to all four stereoisomers of monatin, as well as any blends of any combination of monatin stereoisomers (e.g., a blend of the R,R and S,S, stereoisomers of monatin).

Monatin has an excellent sweetness quality. Monatin has a flavor profile that is as clean or cleaner that other known high intensity sweeteners. The dose response curve of monatin is more linear, and therefore more similar to sucrose than other high intensity sweeteners, such as saccharin. Monatin's excellent sweetness profile makes monatin desirable for use in tabletop sweeteners, foods, beverages and other products.

Different stereoisomers of monatin, including the R,R and S,S stereoisomers, have potential in the sweetener industry, either as separate ingredients or in blends. Monatin, and blends of stereoisomers of monatin with other sweeteners, are thought to have superior taste characteristics and/or physical qualities, as compared to other high intensity sweeteners. For example, monatin is more stable than Equal® or NutraSweet® (aspartame, also known as "APM"), has a cleaner taste than Sweet N'

Low® (saccharin), and is more sweet than Splenda® (sucralose). Likewise, monatin sweeteners do not have the bitter aftertaste associated with saccharin, or the metallic, acidic, astringent or throat burning aftertastes of some other high potency sweeteners. In addition, monatin sweeteners do not exhibit the licorice aftertaste associated with

5   certain natural sweeteners, such as stevioside and glycyrrhizin. Furthermore, unlike aspartame sweeteners, monatin sweeteners do not require a phenylalanine warning for patients with phenylketonuria. Because of its intense sweetness, the R,R stereoisomer in particular should be economically competitive compared to other high intensity sweeteners.

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In one aspect, the present invention provides sweetener compositions containing R,R monatin and/or S,S monatin. For example, such compositions may contain a sweetness comparable to that of granulated sugar (sucrose), and therefore can be used "spoon-for-spoon" or "cup-for-cup" in place of sugar. "A sweetness comparable"

15   means that an experienced sensory evaluator, on average, will determine that the sweetness presented in a first composition is within a range of 80% to 120% of the sweetness presented a second composition. The phrase "a sweetness comparable" relates to a determination ascertained by five or more experienced sensory evaluators in a sweetness matching test, as discussed below, where the test is conducted with

20   compositions (e.g., solutions) having a range of 2-10% sucrose equivalents. Thus, for instance, 100 mg/mL of a composition comprising monatin provides "a sweetness comparable" to 100 mg/mL of sucrose if the monatin composition has a sweetness falling with the range of sweetness presented in 80-120 mg/mL of sucrose.

25   One assesses sweetness of a sweetener relative to sucrose by using a panel of trained sensory evaluators experienced in the sweetness estimation procedure. All samples (in same buffers) are served in duplicate at a temperature of 22°C ± 1°C. Test solutions, coded with 3 digit random number codes, are presented individually to panelists, in random order. Sucrose reference standards, ranging from 4.0 – 10.0%

30   (w/v) sucrose, increasing in steps of 0.5% (w/v) sucrose are also provided. Panelists are asked to estimate sweetness by comparing the sweetness of the test solution to the sucrose standards. This is carried out by taking 3 sips of the test solution, followed by a sip of water, followed by 3 sips of sucrose standard followed by a sip of water, etc.

Panelists estimate the sweetness to one decimal place, e.g., 6.8, 8.5. A five minute rest period is imposed between evaluating the test solutions. Panelists are also asked to rinse well and eat a cracker to reduce any potential carry over effects. Using information obtained from the panelists, the sweetness intensity or potency is

5   calculated by dividing sucrose equivalent value (SEV) (e.g., % sucrose) by the % monatin at a particular point in a dose response curve.

In some embodiments, the compositions comprise monatin that consists essentially of S,S or R,R monatin. In other embodiments, the compositions contain predominantly

10   S,S or R,R monatin. "Predominantly" means that of the monatin sterioisomers present in the composition, the monatin contains greater than 90% of a particular stereoisomer. In some embodiments, the compositions are substantially free of S,S or R,R monatin. "Substantially free" means that of the monatin sterioisomers present in the composition, the composition contains less than 2% of a particular stereoisomer.

15   Additionally or alternatively, when used to describe monatin produced in a biosynthetic pathway, "substantially free" encompasses the amount of a stereoisomer (e.g., S,S monatin) produced as a by-product in a biosynthetic pathway involving chiral-specific enzymes (e.g., D-amino acid dehydrogenases or D-amino acid aminotransferases) and/or chiral-specific substrates (e.g., one having a carbon in the

20   R-stereoconfiguration) to produce a different specific stereostereoisomer (e.g., R,R monatin)

In another aspect of the present invention, a sweetener composition is provided, which includes a stereoisomerically-enriched monatin mixture produced in a biosynthetic

25   pathway. "Stereoisomerically-enriched monatin mixture" means that the mixture contains more than one monatin stereoisomer and at least 60% of the monatin sterioisomers in the mixture is a particular stereoisomer, such as R,R, S,S, S,R or R,S. In other embodiments, the mixture contains greater than 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or 99% of a particular monatin stereoisomer. In another

30   embodiment, a sweetener composition comprises an stereoisomerically-enriched R,R or S,S monatin. "Stereoisomerically-enriched" R,R monatin means that the monatin comprises at least 60% R,R monatin. "Stereoisomerically-enriched" S,S monatin means that the monatin comprises at least 60% S,S monatin. In other embodiments,

"stereoisomerically-enriched" monatin comprises greater than 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98% or 99% of R,R or S,S monatin.

In another aspect, the present invention also provides sweetener formulations, for
5   example, in pre-portioned packets or ready-to-use formulations, which include monatin in the R,R stereoisomer and/or S,S stereoisomer forms. For example, a single serving packet formulation (usually about 1 gram) can provide a sweetness comparable to that contained in two teaspoons of granulated sugar (sucrose). It is known in the art that a "teaspoon" of sucrose contains approximately 4 grams of
10   sucrose. Alternatively, a volume of a ready-to-use formulation can provide a sweetness comparable to the same volume of granulated sugar. Alternatively, a single serving packet of a monatin composition (e.g., 1 gram) can provide a sweetness comparable to about 0.9 to about 9.0 grams of granulated sugar (sucrose). The term "about" encompasses the range of experimental error that occurs in any measurement.
15   Unless otherwise stated, all measurement numbers are presumed to have the word "about" in front of them even if the word "about" is not expressly used.

In another aspect, the present invention provides a homogeneous tabletop sweetener composition comprising monatin. A "homogeneous" composition refers to a uniform
20   composition. For example, a "homogeneous" tabletop sweetener composition (e.g., a liquid) will contain the same concentration of monatin throughout the composition—any sample obtained from that composition will have that concentration.

In another aspect of the present invention, a method of making a sweetener
25   composition is provided. The method includes biosynthetically producing monatin either *in vivo* or *in vitro*. A "biosynthetic pathway" comprises at least one biological conversion step. In some embodiments, the biosynthetic pathway is a multi-step process and at least one step is a biological conversion step. In other embodiments, the biosynthetic pathway is a multi-step process involving both biological and
30   chemical conversion steps. In some embodiments, the monatin produced is a stereoisomerically-enriched monatin mixture.

In another aspect of the present invention, several biosynthetic pathways exist for making monatin from substrates chosen from glucose, tryptophan, indole-3-lactic

acid, as well as indole-3-pyruvate and 2-hydroxy 2-(indole-3-ylmethyl)-4-keto glutaric acid (also known as "the monatin precursor," "MP" or the alpha-keto form of monatin). Examples of biosynthetic pathways for producing or making monatin or its intermediates are disclosed in FIGS. 1-3 and 11-13, which show potential

5   intermediate products and end products in boxes. For example, a conversion from one product to another, such as glucose to tryptophan, tryptophan to indole-3-pyruvate, indole-3-pyruvate to MP, MP to monatin, or indole-3-lactic acid (indole-lactate) to indole-3-pyruvate, occurs in these pathways.

10   These conversions within the biosynthetic pathways can be facilitated via chemical and/or biological conversions. The term "convert" refers to the use of either chemical means or at least one polypeptide in a reaction to change a first intermediate into a second intermediate. Conversions can take place *in vivo* or *in vitro*. The term "chemical conversion" refers to a reaction that is not actively facilitated by a

15   polypeptide. The term "biological conversion" refers to a reaction that is actively facilitated by one or more polypeptides. When biological conversions are used, the polypeptides and/or cells can be immobilized on supports such as by chemical attachment on polymer supports. The conversion can be accomplished using any reactor known to one of ordinary skill in the art, for example in a batch or a

20   continuous reactor.

Examples of polypeptides, and their coding sequences, that can be used to perform biological conversions are shown in FIGS. 1-3 and 11-13. Polypeptides having one or more point mutations that allow the substrate specificity and/or activity of the

25   polypeptides to be modified, can be used to make monatin. Isolated and recombinant cells expressing such polypeptides can be used to produce monatin. These cells can be any cell, such as a plant, animal, bacterial, yeast, algal, archaeal, or fungal cell.

For example, monatin-producing cells can include one or more (such as two or more,

30   or three or more) of the following activities: tryptophan aminotransferase (EC 2.6.1.27), tyrosine (aromatic) aminotransferase (EC 2.6.1.5), tryptophan dehydrogenase (EC 1.4.1.19), glutamate dehydrogenase (EC 1.4.1.2, 1.4.1.3, 1.4.1.4), phenylalanine dehydrogenase (EC 1.4.1.20), tryptophan-phenylpyruvate transaminase (EC 2.6.1.28), multiple substrate aminotransferase (EC 2.6.1.-), aspartate

aminotransferase (EC 2.6.1.1), L-amino acid oxidase (EC 1.4.3.2), tryptophan oxidase (no EC number, Hadar et al., *J. Bacteriol* 125:1096-1104, 1976 and Furuya et al., *Biosci Biotechnol Biochem* 64:1486-93, 2000), D-tryptophan aminotransferase (Kohiba and Mito, Proceedings of the 8th International Symposium on Vitamin B_6 and Carbonyl Catalysis, Osaka, Japan 1990), D-amino acid dehydrogenase (EC 1.4.99.1), D-amino acid oxidase (EC 1.4.3.3), D-alanine aminotransferase (EC 2.6.1.21), synthase/lyase (EC 4.1.3.-), such as 4-hydroxy-2-oxoglutarate aldolase (EC 4.1.3.16) or 4-hydroxy-4-methyl-2-oxoglutarate aldolase (EC 4.1.3.17), and/or synthase/lyase (4.1.2.-).

In another example, cells can include one or more (such as two or more, or three or more) of the following activities: indolelactate dehydrogenase (EC 1.1.1.110), R-4-hydroxyphenyllactate dehydrogenase (EC 1.1.1.222), 3-(4)-hydroxyphenylpyruvate reductase (EC 1.1.1.237), lactate dehydrogenase (EC 1.1.1.27, 1.1.1.28, 1.1.2.3), (3-imidazol-5-yl) lactate dehydrogenase (EC 1.1.1.111), lactate oxidase (EC 1.1.3.-), synthase/lyase (4.1.3.-) such as 4-hydroxy-2-oxoglutarate aldolase (EC 4.1.3.16) or 4-hydroxy-4-methyl-2-oxoglutarate aldolase (EC 4.1.3.17), synthase/lyase (4.1.2.-), tryptophan aminotransferase (EC 2.6.1.27), tyrosine (aromatic) aminotransferase (EC 2.6.1.5), tryptophan dehydrogenase (EC 1.4.1.19), glutamate dehydrogenase (EC 1.4.1.2, 1.4.1.3, 1.4.1.4), phenylalanine dehydrogenase (EC 1.4.1.20), tryptophan-phenylpyruvate transaminase (EC 2.6.1.28), multiple substrate aminotransferase (EC 2.6.1.-), aspartate aminotransferase (EC 2.6.1.1), D-tryptophan aminotransferase, D-amino acid dehydrogenase (EC 1.4.99.1), and/or D-alanine aminotransferase (EC 2.6.1.21).

In addition, the cells can include one or more (such as two or more, or three or more) of the following activities: tryptophan aminotransferase (EC 2.6.1.27), tyrosine (aromatic) aminotransferase (EC 2.6.1.5), tryptophan dehydrogenase (EC 1.4.1.19), glutamate dehydrogenase (EC 1.4.1.2, 1.4.1.3, 1.4.1.4), phenylalanine dehydrogenase (EC 1.4.1.20), tryptophan-phenylpyruvate transaminase (EC 2.6.1.28), multiple substrate aminotransferase (EC 2.6.1.-), aspartate aminotransferase (EC 2.6.1.1), L-amino acid oxidase (EC 1.4.3.2), tryptophan oxidase, D-tryptophan aminotransferase, D-amino acid dehydrogenase (EC 1.4.99.1), D-amino acid oxidase (EC 1.4.3.3), D-alanine aminotransferase (EC 2.6.1.21), indolelactate dehydrogenase (EC 1.1.1.110),

R-4-hydroxyphenyllactate dehydrogenase (EC 1.1.1.222), 3-(4)-
hydroxyphenylpyruvate reductase (EC 1.1.1.237), lactate dehydrogenase (EC
1.1.1.27, 1.1.1.28, 1.1.2.3), (3-imidazol-5-yl) lactate dehydrogenase (EC 1.1.1.111),
lactate oxidase (EC 1.1.3.-), synthase/lyase (EC 4.1.3.-), such as 4-hydroxy-2-

5   oxoglutarate aldolase (EC 4.1.3.16) or 4-hydroxy-4-methyl-2-oxoglutarate aldolase
(EC 4.1.3.17), and/or synthase/lyase
(4.1.2.-).

As further example, the cells can include one or more of the following aldolase

10   activities: KHG aldolase, ProA aldolase, KDPG aldolase and/or related polypeptides
(KDPH), transcarboxybenzalpyruvate hydratase-aldolase, 4-(2-carboxyphenyl)-2-
oxobut-3-enoate aldolase, trans-O-hydroxybenzylidenepyruvate hydratase-aldolase, 3-
hydroxyaspartate aldolase, benzoin aldolase, dihydroneopterin aldolase, L-threo-3-
phenylserine benzaldehyde-lyase (phenylserine aldolase), 4-hydroxy-2-oxovalerate

15   aldolase, 1,2-dihydroxybenzylpyruvate aldolase, and/or 2-hydroxybenzalpyruvate
aldolase.

Monatin can be produced by methods that include contacting tryptophan and/or
indole-3-lactic acid with a first polypeptide, wherein the first polypeptide converts

20   tryptophan and/or indole-3-lactic acid to indole-3-pyruvate (either the D or the L form
of tryptophan or indole-3-lactic acid can be used as the substrate that is converted to
indole-3-pyruvate; one of skill in the art will appreciate that the polypeptides chosen
for this step ideally exhibit the appropriate specificity), contacting the resulting
indole-3-pyruvate with a second polypeptide, wherein the second polypeptide

25   converts the indole-3-pyruvate to 2-hydroxy 2-(indol-3-ylmethyl)-4-keto glutaric acid
(MP), and contacting the MP with a third polypeptide, wherein the third polypeptide
converts MP to monatin. Exemplary polypeptides that can be used for these
conversions are shown in FIGS. 2 and 3.

30   Producing monatin in a biosynthetic pathway via one or more biological conversions
provides certain advantages. For example, by using specific polypeptides and/or
certain substrates in the biosynthetic pathway, one can produce a mixture enriched in
a specific stereoisomer, and/or produce a monatin mixture that is substantially free of
one or more stereoisomers.

A monatin composition may include impurities as a consequence of the method used for monatin synthesis. Monatin compositions produced by purely synthetic means (i.e., not involving at least one biological conversion) will contain different impurities than monatin compositions produced via a biosynthetic pathway. For example, based on raw materials used, monatin compositions produced by purely synthentic means may include petrochemical, toxic and/or other hazardous contaminants inappropriate for human consumption. Examples of such contaminants are hazardous chemicals, such as LDA, hydrogen-Pd/C, diazomethane, KCN, Grignard's reagent and Na/Hg. On the other hand, it is expected that a monatin composition produced via a biosynthetic pathway may contain edible or potable impurities, but will not contain petrochemical, toxic and/or other hazardous material.

It is expected that a method for producing monatin in a biosynthetic pathway via one or more biological conversions produces fewer toxic or hazardous contaminants and/or can provide a higher percentage of a particular stereoisomer, as compared to purely synthetic means. For example, it is expected that when making monatin using D-amino acid dehydrogenases, D-alanine (aspartate) aminotransferases, D-aromatic aminotransferases or D-methionine aminotranferases, one can obtain at least 60% R,R monatin and less than 40 % S,S, S,R and/or R,S monatin. It is also expected, for example, that when making monatin using the above-mentioned D-enzymes, as well as at least one substrate (e.g., the monatin precursor) having a carbon in the R-stereoconfiguration, one can obtain at least 95 % R,R monatin and less than 5% S,S, S,R and/or R,S monatin. In contrast, it is expected that when making monatin by purely synthetic means, one obtains about 25%-50% of the desired stereoisomer.

In one embodiment, a method for producing monatin via a biosynthetic pathway, for example, involving one or more biological conversion, produces no petrochemical, toxic or hazardous contaminants. "Petrochemical, toxic or hazardous contaminants" means any material that is petrochemical, toxic, hazardous and/or otherwise inappropriate for human consumption, including those contaminants provided as raw material or created when producing monatin via purely synthetic means. In another embodiment, a method for producing monatin via a biosynthetic pathway, for example, involving one or more biological conversion, produces only edible or

potable material. "Edible or potable material" means one or more compounds or material that are fit for eating or drinking by humans, or otherwise safe for human consumption. Examples of edible or potable material include monatin, tryptophan, pyruvate, glutamate, other amino acids, as well as other compounds or material that are naturally present in the body.

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In certain embodiments, monatin sweetener compositions include tabletop pre-portioned packets and cubes of sweetener, as well as tabletop ready-to-use "spoon-for-spoon" sweeteners having approximately the same sweetness as sucrose (sugar) by volume, which can be used as a substitute for tabletop granulated sugar. Such tabletop sweeteners may be used to sweeten coffee and tea (both hot and iced), cereal, fruit and home baked goods (cookies, muffins, cakes, etc.) and desserts.

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In one embodiment, a tabletop sweetener composition comprises monatin or salt thereof, wherein the composition provides a sweetness comparable to about 0.9 to about 9.0 grams of granulated sugar. In another embodiment, a 1 gram portion of the monatin tabletop sweetener composition provides a sweetness comparable to two teaspoons of granulated sugar. In another embodiment, a 1 gram portion of the monatin tabletop sweetener composition provides provides a sweetness comparable to about 0.9 to about 9.0 grams of granulated sugar, and contains less calories and carbohydrates than about 1 gram of granulated sugar.

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In other embodiments, a tabletop sweetener composition comprising monatin or salt thereof comprises from about 0 to about 200 mg S,S monatin or salt thereof, and from about 0 to about 5 mg R,R monatin or salt thereof. For example, 1 gram of the tabletop sweetener composition may comprise from about 3 to about 200 mg S,S monatin or salt thereof, and from about 0 to about 5 mg R,R monatin or salt thereof. Alternatively, for example, 1 gram of the tabletop sweetener composition may comprise from about 0 to about 200 mg S,S monatin or salt thereof, and from about 3 to about 5 mg R,R monatin or salt thereof.

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In other embodiments, 1 gram of the monatin tabletop sweetener composition comprises about 200 mg or less S,S monatin or salt thereof, and is substantially free of R,R, S,R or R,S monatin or salt thereof. In another embodiments, 1 gram of the

monatin tabletop sweetener composition comprises about 5 mg or less R,R monatin or salt thereof, and is substantially free of S,S, S,R or R,S monatin or salt thereof.

In other embodiments, composition comprising monatin or salt thereof further
5   comprise at least one ingredient chosen from bulking agents, carriers, fibers, sugar alcohols, oligosaccharides, sugars, non-monatin high intensity sweeteners, nutritive sweeteners, flavorings, flavor enhancers, flavor stablizers, acidulants, anti-caking agents, and free-flow agents. For example, the monatin tabletop sweetener compositions may further comprise agglomerated dextrose with maltodextrin

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In one embodiment, a sweetener composition comprises monatin and erthritol. In other embodiments, a monatin composition comprises erythritol. In one embodiment, a monatin composition comprises up to 99.7% erythritol. In another embodiment, a monatin composition comprises trehalose. In another embodiment, a monatin
15   composition comprises cyclamate.

In other embodiments, a tabletop sweetener composition comprises monatin or salt thereof, where the monatin or salt thereof consists essentially of R,R monatin or salt thereof. Alternatively, the tabletop sweetener composition comprises monatin or salt
20   thereof, wherein the monatin or salt thereof consists essentially of S,S monatin or salt thereof. In other embodiments, a tabletop sweetener composition comprises monatin or salt thereof, where the the monatin or salt thereof is a stereoisomerically-enriched R,R or S,S monatin or salt thereof. For example, the monatin tabletop sweetener composition may comprises at least 95% R,R monatin or salt thereof.

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In other embodiments, a tabletop sweetener composition comprises monatin or salt thereof, wherein the sweetness is provided by monatin or salt thereof produced in a biosynthetic pathway.

30   In other embodiments, a ready-to-use sweetener composition comprises monatin or salt thereof, wherein a volume of the composition provides a sweetness comparable to a same volume of granulated sugar. In another embodiment, 1 teaspoon of the monatin ready-to-use sweetener composition contains less calories and carbohydrates than about 1 teaspoon of granulated sugar.

In other embodiments, a ready-to-use sweetener composition comprises monatin or salt thereof, wherein 1 gram of the composition comprises about 3 to about 25 mg S,S monatin or salt thereof, and from about 0 to about 0.625 mg R,R monatin or salt thereof. Alternatively, for example, the monatin ready-to-use sweetener consists essentially of S,S monatin or salt thereof or R,R monatin or salt thereof. In other embodiments, 1 gram of a ready-to-use monatin sweetener composition comprises from about 5 to about 25 mg S,S monatin or salt thereof. Alternatively, for example, 1 gram of a ready-to-use monatin sweetener composition comprises from about 0.4 to about 0.625 R,R monatin or salt thereof, and is substantially free of S,S, S,R or R,S monatin or salt thereof. Alternatively, for example, 1 gram of a ready-to-use monatin sweetener composition comprises from about 0.5 to about 1 mg R,R monatin or salt thereof, and is substantially free of S,S, S,R or R,S monatin or salt thereof.

In other embodiments, a sweetener composition comprises a stereoisomerically-enriched monatin mixture, wherein the monatin mixture is produced via a biosynthetic pathway. In one embodiment, the biosynthetic pathway is a multi-step pathway and at least one step of the multi-step pathway is a chemical conversion. In another embodiment, the stereoisomerically-enriched monatin mixture is predominantly R,R monatin or salt thereof.

In other embodiments, a homogeneous tabletop sweetener composition comprises monatin or salt thereof, wherein a sample of the composition comprises monatin or salt thereof in an amount ranging from more than 2 mg to about 200 mg, and wherein the monatin or salt thereof in the sample provides a sweetness comparable to about 0.9 to about 9.0 grams of granulated sugar. For example, a sample of the homogeneous tabletop sweetener composition may provide a sweetness comparable to two teaspoons of granulated sugar. In another embodiment, a sample of the homogeneous tabletop sweetener composition weighs about 1 gram or has a volume of about 0.35 mL. In another embodiment, a sample of the homogeneous tabletop sweetener composition comprises an amount of monatin or salt thereof ranging from more than 2 mg to about 5 mg monatin or salt thereof. In another embodiment, the homogeneous tabletop sweetener composition is substantially free of S,S monatin or salt thereof, or substantially free of R,R monatin or salt thereof.

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In other embodiments, a tabletop sweetener composition comprises monatin or salt thereof, wherein a sample of the composition comprises monatin or salt thereof in an amount ranging from more than 2 mg to about 105 mg monatin or salt thereof, and
5   wherein the monatin or salt thereof in the sample provides a sweetness comparable to one teaspoon of granulated sugar. In another embodiment, a sample of the tabletop sweetener composition comprising monatin or salt thereof has a volume of about 0.35 ml, or is a cube of granulated material.

10   In other embodiments, a tabletop sweetener composition comprises a monatin composition produced in a biosynthetic pathway, wherein the monatin composition does not contain petrochemical, toxic or hazardous contaminants. For example, the monatin tabletop sweetener composition comprising monatin or salt thereof may be produced in a biosynthetic pathway and isolated from a recombinant cell, wherein the
15   recombinant cell does not contain petrochemical, toxic or hazardous contaminants.

In other embodiments, a method for making a sweetener composition comprises monatin or salt thereof, wherein the method comprises producing monatin or salt thereof from at least one substrate chosen from glucose, tryptophan, indole-3-lactic
20   acid, indole-3-pyruvate and the monatin precursor. In other embodiments, the method further comprises combining the monatin or salt thereof with erythritol. In other embodiments, the method further comprises combining the monatin or salt thereof with trehalose. In other embodiments, the method further comprises combining the monatin or salt thereof with cyclamate. In other embodiments, the method further
25   comprises combining the monatin or salt thereof with at least one other ingredient that is not monatin or salt thereof. Such ingredients may include bulking agents, carriers, fibers, sugar alcohols, oligosaccharides, sugars, non-monatin high intensity sweeteners, nutritive sweeteners, flavorings, flavor enhancers, flavor stabilizers, acidulants, anti-caking, free-flow agents, and any combination thereof. In one
30   embodiment, at least one other ingredient is chosen from maltodextrin, dextrose, erythritol and fiber.

In other embodiments, in a method for making a sweetener composition, a portion of the sweetener composition weighing about 1 gram comprises from about 0 mg to

about 200 mg S,S monatin or salt thereof and from about 0 mg to about 5 mg R,R monatin or salt thereof, and the portion provides a sweetness comparable to two teaspoons of granulated sugar.

5   In other embodiments, in a method for making a sweetener composition, 1 gram of the sweetener composition comprises from about 0 to about 25 mg S,S monatin or salt thereof and from about 0 to about 0.625 mg R,R monatin or salt thereof, wherein a volume of the composition has a sweetness comparable to a same volume of granulated sugar. Alternatively, in a method for making a sweetener composition, 1

10   gram of the composition comprises from about 0 to about 25 mg S,S monatin or salt thereof and from about 0 to about 0.625 mg R,R monatin or salt thereof, wherein 1 gram of the composition has a sweetness comparable to about 0.9 to about 9.0 grams granulated sugar. Alternatively, in a method for making a sweetener composition, the amount of S,S monatin or salt thereof ranges from about 5 to about 200 mg S,S

15   monatin or salt thereof per 1 gram of the composition.

In other embodiments, a method for making a sweetener composition further comprises combining S,S monatin or salt thereof with at least one other ingredient chosen from bulking agents, carriers, fibers, sugar alcohols, oligosaccharides, sugars,

20   non-monatin high intensity sweeteners, nutritive sweeteners, flavorings, flavor enhancers, flavor stablizers, acidulants, anti-caking, free-flow agents, and any combination thereof, wherein the monatin or salt thereof comprises about 3 to about 200 mg of monatin or salt thereof per 1 gram of the composition. In other embodiments, a method for making a sweetener composition further comprises

25   combining R,R monatin or salt thereof with at least one other ingredient chosen from bulking agents, carriers, fibers, sugar alcohols, oligosaccharides, sugars, non-monatin high intensity sweeteners, nutritive sweeteners, flavorings, flavor enhancers, flavor stablizers, acidulants, anti-caking, free-flow agents, and any combination thereof, wherein the amount of R,R monatin or salt thereof ranges from about 3 to about 5 mg

30   of R,R monatin or salt thereof per 1 gram of the composition. In other embodiments, a method for making a sweetener composition comprises from about 0.4 to about 5 mg R,R monatin or salt thereof per 1 gram of the composition.

In other embodiments, a method for making a sweetener composition comprises combining monatin or salt thereof with at least one bulking agent chosen from dextrose and maltodextrin, and wherein the monatin or salt thereof comprises about 5 mg R,R monatin or salt thereof per 1 gram of the composition. In other embodiments,

5   a method for making a sweetener composition comprises combining monatin or salt thereof with maltodextrin, and wherein the monatin or salt thereof comprises from about 0.5 to about 1 mg R,R monatin or salt thereof per 1 gram of the composition.

In other embodiments, in a method for making a sweetener composition comprising a

10   monatin composition, the method comprises producing the monatin composition in a biosynthetic pathway, and wherein the monatin composition does not contain petrochemical, toxic or hazardous contaminants. Alternatively, the method comprises producing the monatin composition from a substrate in a multi-step pathway, wherein one or more steps in the multi-step pathway is a biological

15   conversion, and wherein the monatin composition does not contain petrochemical, toxic or hazardous contaminants.

In other embodiments, in a method for making a sweetener composition comprising a monatin composition, the method comprises producing the monatin composition in a

20   biosynthetic pathway, and wherein the monatin composition consists of monatin or salt thereof and other edible or potable material. Alternatively, the method comprises producing the monatin composition from a substrate in a multi-step pathway, wherein one or more steps in the multi-step pathway is a biological conversion, and wherein the monatin composition consists of monatin or salt thereof and other edible or

25   potable material.

In other embodiments, in a method for making a sweetener composition comprising a monatin composition, the method comprises: (a) producing monatin or salt thereof in a biosynthetic pathway in a recombinant cell; (b) isolating the monatin composition

30   from the recombinant cell, wherein the monatin composition consists of monatin or salt thereof and other edible or potable material.

It will be apparent to one of ordinary skill in the art from the teachings herein that specific embodiments of the present invention may be directed to one or a combination of the above-indicated aspects, as well as other aspects.

5

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 shows biosynthetic pathways used to produce monatin and/or indole-3-pyruvate. One pathway produces indole-3-pyruvate via tryptophan, while another
10 produces indole-3-pyruvate via indole-3-lactic acid. Monatin is subsequently produced via a MP intermediate.

Compounds shown in boxes are substrates and products produced in the biosynthetic pathways. Compositions adjacent to the arrows are cofactors, or reactants that can be
15 used during the conversion of a substrate to a product. The cofactor or reactant used will depend upon the polypeptide used for the particular step of the biosynthetic pathway. The cofactor PLP (pyridoxal 5' – phosphate) can catalyze reactions independent of a polypeptide, and therefore, merely providing PLP can allow for the progression from substrate to product.

20
FIG. 2 is a more detailed diagram of the biosynthetic pathway that utilizes the MP intermediate. The substrates for each step in the pathways are shown in boxes. The polypeptides allowing for the conversion between substrates are listed adjacent to the arrows between the substrates. Each polypeptide is described by its common name
25 and an enzymatic class (EC) number.

FIG. 3 shows a more detailed diagram of the biosynthetic pathway of the conversion of indole-3-lactic acid to indole-3-pyruvate. The substrates are shown in boxes, and the polypeptides allowing for the conversion between the substrates are listed adjacent
30 to the arrow between the substrates. Each polypeptide is described by its common name and an EC number.

FIG. 4 shows one possible reaction for making MP via chemical means.

FIGS. 5A and 5B are chromatograms showing the LC/MS identification of monatin produced enzymatically.

FIG. 6 is an electrospray mass spectrum of enzymatically synthesized monatin.

FIGS. 7A and 7B are chromatograms of the LC/MS/MS daughter ion analyses of monatin produced in an enzymatic mixture.

FIG. 8 is a chromatogram showing the high-resolution mass measurement of monatin produced enzymatically.

FIGS. 9A-9C are chromatograms showing the chiral separation of (A) R-tryptophan, (B) S-tryptophan, and (C) monatin produced enzymatically.

FIG. 10 is a bar graph showing the relative amount of monatin produced in bacterial cells following IPTG induction. The (-) indicates a lack of substrate addition (no tryptophan or pyruvate was added).

FIGS. 11-12 are schematic diagrams showing pathways used to increase the yield of monatin produced from tryptophan or indole-3-pyruvate.

FIG. 13 is a schematic diagram showing a pathway that can be used to increase the yield of monatin produced from tryptophan or indole-3-pyruvate.

FIG. 14 presents a dose response curve obtained with an R,R, stereoisomer of monatin.

FIG. 15 presents a dose response curve obtained with an R,R/S,S stereoisomer mixture of monatin.

FIG. 16 compares the dose response curve obtained with an R,R/S,S stereoisomer mixture of monatin to a dose response curve obtained with saccharin.

FIG. 17 shows reversed phase chromatography of standards of synthetically produced monatin.

FIG. 18 shows chiral chromatography of monatin standards.

5

DETAILED DESCRIPTION

Overview of Biosynthetic pathways for Monatin Production

10   The following explanations of terms and methods are provided to better describe the present disclosure and to guide those of ordinary skill in the art in the practice of the present disclosure. As used herein, "including" means "comprising." In addition, the singular forms "a" or "an" or "the" include plural references unless the context clearly dictates otherwise.

15

As shown in FIGS. 1–3 and 11-13, many biosynthetic pathways can be used to produce monatin or its intermediates such as indole-3-pyruvate or MP. For the conversion of each substrate (e.g., glucose, tryptophan, indole-3-lactic acid, indole-3-pyruvate, and MP) to each product (e.g., tryptophan, indole-3-pyruvate, MP and

20   monatin), several different polypeptides can be used. Moreover, these reactions can be carried out *in vivo*, *in vitro*, or through a combination of *in vivo* reactions and *in vitro* reactions, such as *in vitro* reactions that include non-enzymatic chemical reactions. Therefore, FIGS. 1-3 and 11-13 are exemplary, and show multiple different pathways that can be used to obtain desired products.

25

Glucose to Tryptophan
Many organisms can synthesize tryptophan from glucose. The construct(s) containing the gene(s) necessary to produce monatin, MP, and/or indole-3-pyruvate from glucose and/or tryptophan can be cloned into such organisms. It is shown herein that

30   tryptophan can be converted into monatin.

In other examples, an organism can be engineered using known polypeptides to produce tryptophan, or overproduce tryptophan. For example, U.S. Patent No.

4,371,614 describes an *E. coli* strain transformed with a plasmid containing a wild type tryptophan operon.

5   Maximum titers of tryptophan disclosed in U.S. Patent No. 4,371,614 are about 230 ppm. Similarly, WO 8701130 describes an *E. coli* strain that has been genetically engineered to produce tryptophan and discusses increasing fermentative production of L-tryptophan. Those skilled in the art will recognize that organisms capable of producing tryptophan from glucose are also capable of utilizing other carbon and energy sources that can be converted to glucose or fructose-6-phosphate, with similar
10   results. Exemplary carbon and energy sources include, but are not limited to, sucrose, fructose, starch, cellulose, or glycerol.

Tryptophan to Indole-3-pyruvate

Several polypeptides can be used to convert tryptophan to indole-3-pyruvate.
15   Exemplary polypeptides include, without limitation, members of the enzyme classes (EC) 2.6.1.27, 1.4.1.19, 1.4.99.1, 2.6.1.28, 1.4.3.2, 1.4.3.3, 2.6.1.5, 2.6.1.-, 2.6.1.1, and 2.6.1.21. These classes include, without limitation, polypeptides termed tryptophan aminotransferase (also termed L-phenylalanine-2-oxoglutarate aminotransferase, tryptophan transaminase, 5-hydroxytryptophan-ketoglutaric
20   transaminase, hydroxytryptophan aminotransferase, L-tryptophan aminotransferase, L-tryptophan transaminase, and L-tryptophan:2-oxoglutarate aminotransferase) which converts L-tryptophan and 2-oxoglutarate to indole-3-pyruvate and L-glutamate; D-tryptophan aminotransferase which converts D-tryptophan and a 2-oxo acid to indole-3-pyruvate and an amino acid; tryptophan dehydrogenase (also termed NAD(P)-L-
25   tryptophan dehydrogenase, L-tryptophan dehydrogenase, L-Trp-dehydrogenase, TDH and L-tryptophan:NAD(P) oxidoreductase (deaminating)) which converts L-tryptophan and NAD(P) to indole-3-pyruvate and NH_3 and NAD(P)H; D-amino acid dehydrogenase, which converts D-amino acids and FAD to indole-3-pyruvate and NH_3 and $FADH_2$; tryptophan-phenylpyruvate transaminase (also termed L-
30   tryptophan-α-ketoisocaproate aminotransferase and L-tryptophan:phenylpyruvate aminotransferase) which converts L-tryptophan and phenylpyruvate to indole-3-pyruvate and L-phenylalanine; L-amino acid oxidase (also termed ophio-amino-acid oxidase and L-amino-acid:oxygen oxidoreductase (deaminating)) which converts an L-amino acid and H_2O and O_2 to a 2-oxo acid and NH_3 and H_2O_2; D-amino acid

oxidase (also termed ophio-amino-acid oxidase and D-amino-acid:oxygen

oxidoreductase (deaminating)) which converts a D-amino acid and H_2O and O_2 to a 2-

oxo acid and NH_3 and H_2O_2; and tryptophan oxidase which converts L-tryptophan and

H_2O and O_2 to indole-3-pyruvate and NH_3 and H_2O_2. These classes also contain

5   tyrosine (aromatic) aminotransferase, aspartate aminotransferase, D-amino acid (or D-

alanine) aminotransferase, and broad (multiple substrate) aminotransferase which

have multiple aminotransferase activities, some of which can convert tryptophan and

a 2-oxo acid to indole-3-pyruvate and an amino acid.

10   Eleven members of the aminotransferase class that have such activity are described

below in Example 1, including a novel aminotransferase shown in SEQ ID NOS: 11

and 12. Therefore, this disclosure provides isolated nucleic acid and amino acid

sequences having at least 80%, at least 85%, at least 90%, at least 95%, at least 98%,

or even at least 99% sequence identity to the sequences set forth in SEQ ID NOS: 11

15   and 12, respectively. Also encompassed by this disclosure are fragments and fusions

of the sequences set forth in SEQ ID NOS: 11 and 12 that encode a polypeptide

having aminotransferase activity or retaining aminotransferase activity. Exemplary

fragments include, but are not limited to, at least 10, 12, 15, 20, 25, 50, 100, 200, 500,

or 1000 contiguous nucleotides of SEQ ID NO: 11 or at least 6, 10, 15, 20, 25, 50, 75,

20   100, 200, 300 or 350 contiguous amino acids of SEQ ID NO: 12. The disclosed

sequences (and variants, fragments, and fusions thereof) can be part of a vector. The

vector can be used to transform host cells, thereby producing recombinant cells which

can produce indole-3-pyruvate from tryptophan, and in some examples can further

produce MP and/or monatin.

25

L-amino acid oxidases (1.4.3.2) are known, and sequences can be isolated from

several different sources, such as *Vipera lebetine* (sp P81375), *Ophiophagus hannah*

(sp P81383), *Agkistrodon rhodostoma* (spP81382), *Crotalus atrox* (sp P56742),

Burkholderia cepacia, Arabidopsis thaliana, Caulobacter cresentus, Chlamydomonas

30   *reinhardtii, Mus musculus, Pseudomonas syringae,* and *Rhodococcus* str. In addition,

tryptophan oxidases are described in the literature and can be isolated, for example,

from *Coprinus* sp. SF-1, Chinese cabbage with club root disease, *Arabidopsis*

thaliana, and mammalian liver. One member of the L-amino acid oxidase class that

can convert tryptophan to indole-3-pyruvate is discussed below in Example 3, as well

as alternative sources for molecular cloning. Many D-amino acid oxidase genes are available in databases for molecular cloning.

5   Tryptophan dehydrogenases are known, and can be isolated, for example, from spinach, *Pisum sativum*, *Prosopis juliflora*, pea, mesquite, wheat, maize, tomato, tobacco, *Chromobacterium violaceum*, and *Lactobacilli*. Many D-amino acid dehydrogenase gene sequences are known.

       As shown in FIGS. 11-13, if an amino acid oxidase, such as tryptophan oxidase, is
10   used to convert tryptophan to indole-3-pyruvate, catalase can be added to reduce or even eliminate the presence of hydrogen peroxide.

Indole-3-lactate to Indole-3-pyruvate

       The reaction that converts indole-3-lactate to indole-3-pyruvate can be catalyzed by a
15   variety of polypeptides, such as members of the 1.1.1.110, 1.1.1.27, 1.1.1.28, 1.1.2.3, 1.1.1.222, 1.1.1.237, 1.1.3.-, or 1.1.1.111 classes of polypeptides. The 1.1.1.110 class of polypeptides includes indolelactate dehydrogenases (also termed indolelactic acid: NAD^+ oxidoreductase). The 1.1.1.27, 1.1.1.28, and 1.1.2.3 classes include lactate dehydrogenases (also termed lactic acid dehydrogenases, lactate: NAD^+
20   oxidoreductase). The 1.1.1.222 class contains (R)-4-hydroxyphenyllactate dehydrogenase (also termed D-aromatic lactate dehydrogenase, R-aromatic lactate dehydrogenase, and R-3-(4-hydroxyphenyl)lactate:$NAD(P)^+$ 2-oxidoreductase) and the 1.1.1.237 class contains 3-(4-hydroxyphenylpyruvate) reductase (also termed hydroxyphenylpyruvate reductase and 4-hydroxyphenyllactate: NAD^+
25   oxidoreductase). The 1.1.3.- class contains lactate oxidases, and the 1.1.1.111 class contains (3-imidazol-5-yl) lactate dehydrogenases (also termed (S)-3-(imidazol-5-yl)lactate:$NAD(P)^+$ oxidoreductase). It is likely that several of the polypeptides in these classes allow for the production of indole-3-pyruvate from indole-3-lactic acid. Examples of this conversion are provided in Example 2.
30

       Chemical reactions can also be used to convert indole-3-lactic acid to indole-3-pyruvate. Such chemical reactions include an oxidation step that can be accomplished using several methods, for example: air oxidation using a B2 catalyst (China

Chemical Reporter, vol. 13, no. 28, pg. 18(1), 2002), dilute permanganate and perchlorate, or hydrogen peroxide in the presence of metal catalysts.

Indole-3-pyruvate to 2-hydroxy 2-(indol-3ylmethyl)-4-keto glutaric acid (MP)

5   Several known polypeptides can be used to convert indole-3-pyruvate to MP. Exemplary polypeptide classes include 4.1.3.-, 4.1.3.16, 4.1.3.17, and 4.1.2.-. These classes include carbon-carbon synthases/lyases, such as aldolases that catalyze the condensation of two carboxylic acid substrates. Polypeptide class EC 4.1.3.- are synthases/lyases that form carbon-carbon bonds utilizing oxo-acid substrates (such as

10   indole-3-pyruvate) as the electrophile, while EC 4.1.2.- are synthases/lyases that form carbon-carbon bonds utilizing aldehyde substrates (such as benzaldehyde) as the electrophile.

For example, the polypeptide described in EP 1045-029 (EC 4.1.3.16, 4-hydroxy-2-

15   oxoglutarate glyoxylate-lyase also termed 4-hydroxy-2-oxoglutarate aldolase, 2-oxo-4-hydroxyglutarate aldolase or KHG aldolase) converts glyoxylic acid and pyruvate to 4-hydroxy-2-ketoglutaric acid, and the polypeptide 4-hydroxy-4-methyl-2-oxoglutarate aldolase (EC 4.1.3.17, also termed 4-hydroxy-4-methyl-2-oxoglutarate pyruvate-lyase or ProA aldolase), condenses two keto-acids such as two pyruvates to

20   4-hydroxy-4-methyl-2-oxoglutarate. Reactions utilizing these lyases are described herein.

FIGS. 1-2 and 11-13 show schematic diagrams of these reactions in which a 3-carbon (C3) molecule is combined with indole-3-pyruvate. Many members of EC 4.1.2.- and

25   4.1.3.-, particularly PLP-utilizing polypeptides, can utilize C3 molecules that are amino acids such as serine, cysteine, and alanine, or derivatives thereof. Aldol condensations catalyzed by representatives of EC 4.1.2.- and 4.1.3.- require the three carbon molecule of this pathway to be pyruvate or a derivative of pyruvate. However, other compounds can serve as a C3 carbon source and be converted to pyruvate.

30   Alanine can be transaminated by many PLP-utilizing transaminases, including many of those mentioned above, to yield pyruvate. Pyruvate and ammonia can be obtained by beta-elimination reactions (such as those catalyzed by tryptophanase or β-tyrosinase) of L-serine, L-cysteine, and derivatives of serine and cysteine with sufficient leaving groups, such as O-methyl-L-serine, O-benzyl-L-serine, S-

methylcysteine, S-benzylcysteine, S-alkyl-L-cysteine, O-acyl-L-serine, and 3-chloro-L-alanine. Aspartate can serve as a source of pyruvate in PLP-mediated beta-lyase reactions such as those catalyzed by tryptophanase (EC 4.1.99.1) and/or β-tyrosinase (EC 4.1.99.2, also termed tyrosine-phenol lyase). The rate of beta-lyase reactions can

5   be increased by performing site-directed mutagenesis on the (4.1.99.1-2) polypeptides as described by Mouratou et al. (J. Biol. Chem 274:1320-5, 1999) and in Example 8. These modifications allow the polypeptides to accept dicarboxylic amino acid substrates. Lactate can also serve as a source of pyruvate, and is oxidized to pyruvate by the addition of lactate dehydrogenase and an oxidized cofactor or lactate oxidase

10 and oxygen. Examples of these reactions are described below. For example, as shown in FIG. 2 and FIGS. 11-13, ProA aldolase can be contacted with indole-3-pyruvate when pyruvate is used as the C3 molecule.

The MP can also be generated using chemical reactions, such as the aldol

15 condensations provided in Example 5.

MP to Monatin

Conversion of MP to monatin can be catalyzed by one or more of: tryptophan aminotransferases (2.6.1.27), tryptophan dehydrogenases (1.4.1.19), D-amino acid

20 dehydrogenases (1.4.99.1), glutamate dehydrogenases (1.4.1.2-4), phenylalanine dehydrogenase (EC 1.4.1.20), tryptophan-phenylpyruvate transaminases (2.6.1.28), or more generally members of the aminotransferase family (2.6.1.-) such as aspartate aminotransferase (EC 2.6.1.1), tyrosine (aromatic) aminotransferase (2.6.1.5), D-tryptophan aminotransferase, or D-alanine (2.6.1.21) aminotransferase (FIG. 2).

25 Eleven members of the aminotransferase class are described below (Example 1), including a novel member of the class shown in SEQ ID NOS: 11 and 12, and reactions demonstrating the activity of aminotransferase and dehydrogenase enzymes are provided in Example 7.

30 This reaction can also be performed using chemical reactions. Amination of the keto acid (MP) is performed by reductive amination using ammonia and sodium cyanoborohydride.

FIGS. 11-13 show additional polypeptides that can be used to convert MP to monatin, as well as providing increased yields of monatin from indole-3-pyruvate or tryptophan. For example, if aspartate is used as the amino donor, aspartate aminotransferase can be used to convert the aspartate to oxaloacetate (FIG. 11). The

5   oxaloacetate is converted to pyruvate and carbon dioxide by a decarboxylase, such as oxaloacetate decarboxylase (FIG. 11). In addition, if lysine is used as the amino donor, lysine epsilon aminotransferase can be used to convert the lysine to allysine (FIG. 12). The allysine is spontaneously converted to 1-piperideine 6-carboxylate (FIG. 12). If a polypeptide capable of catalyzing reductive amination reactions (e.g.,

10   glutamate dehydrogenase) is used to convert MP to monatin, a polypeptide that can recycle NAD(P)H and/or produce a volatile product (FIG. 13) can be used, such as formate dehydrogenase.

Additional Considerations in the Design of the Biosynthetic Pathways

15   Depending on which polypeptides are used to generate indole-3-pyruvate, MP, and/or monatin, cofactors, substrates, and/or additional polypeptides can be provided to the production cell to enhance product formation. In addition, genetic modification can be designed to enhance production of products such as indole-3-pyruvate, MP, and/or monatin. Similarly, a host cell used for monatin production can be optimized.

20

Removal of Hydrogen Peroxide

Hydrogen peroxide (H_2O_2) is a product that, if generated, can be damaging to production cells, polypeptides or products (e.g., intermediates) produced. The L-amino acid oxidase described above generates H_2O_2 as a product. Therefore, if L-

25   amino acid oxidase is used, the resulting H_2O_2 can be removed or its levels decreased to reduce potential injury to the cell or product.

Catalases can be used to reduce the level of H_2O_2 in the cell (FIGS. 11-13). The production cell can express a gene or cDNA sequence that encodes a catalase (EC

30   1.11.1.6), which catalyzes the decomposition of hydrogen peroxide into water and oxygen gas. For example, a catalase can be expressed from a vector transfected into the production cell. Examples of catalases that can be used include, but are not limited to: tr|Q9EV50 (*Staphylococcus xylosus*), tr|Q9KBE8 (*Bacillus halodurans*), tr|Q9URJ7 (*Candida albicans*), tr|P77948 (*Streptomyces coelicolor*), tr|Q9RBJ5

(*Xanthomonas campestris*) (SwissProt Accession Nos.). Biocatalytic reactors utilizing L-amino acid oxidase, D-amino acid oxidase, or tryptophan oxidase can also contain a catalase polypeptide.

5   *Modulation of pyridoxal-5'-phosphate (PLP) Availability*
     As shown in FIG. 1, PLP can be utilized in one or more of the biosynthetic steps described herein. The concentration of PLP can be supplemented so that PLP does not become a limitation on the overall efficiency of the reaction.

10   The biosynthetic pathway for vitamin B_6 (the precursor of PLP) has been thoroughly studied in *E. coli*, and some of the proteins have been crystallized (Laber *et al.*, *FEBS Letters*, 449:45-8, 1999). Two of the genes (*epd* or *gapB* and *serC*) are required in other metabolic pathways, while three genes (*pdxA*, *pdxB*, and *pdxJ*) are unique to pyridoxal phosphate biosynthesis. One of the starting materials in the *E. coli* pathway
15   is 1-deoxy-D-xylulose-5-phosphate (DXP). Synthesis of this precursor from common 2 and 3 carbon central metabolites is catalyzed by the polypeptide 1-deoxy-D-xylulose-5-phosphate synthase (DXS). The other precursor is a threonine derivative formed from the 4-carbon sugar, D-erythrose 4-phosphate. The genes required for the conversion to phospho-4-hydroxyl-L threonine (HTP) are *epd*, *pdxB*, and *serC*. The
20   last reaction for the formation of PLP is a complex intramolecular condensation and ring-closure reaction between DXP and HTP, catalyzed by the gene products of *pdxA* and *pdxJ*.

     If PLP becomes a limiting nutrient during the fermentation to produce monatin,
25   increased expression of one or more of the pathway genes in a production host cell can be used to increase the yield of monatin. A host organism can contain multiple copies of its native pathway genes or copies of non-native pathway genes can be incorporated into the organism's genome. Additionally, multiple copies of the salvage pathway genes can be cloned into the host organism.
30
     One salvage pathway that is conserved in all organisms recycles the various derivatives of vitamin B_6 to the active PLP form. The polypeptides involved in this pathway are *pdxK* kinase, *pdxH* oxidase, and *pdxY* kinase. Over-expression of one or more of these genes can increase PLP availability.

25

Vitamin B_6 levels can be elevated by elimination or repression of the metabolic regulation of the native biosynthetic pathway genes in the host organism. PLP represses polypeptides involved in the biosynthesis of the precursor threonine

5   derivative in the bacterium *Flavobacterium sp.* strain 238-7. This bacterial strain, freed of metabolic control, overproduces pyridoxal derivatives and can excrete up to 20 mg/L of PLP. Genetic manipulation of the host organism producing monatin in a similar fashion will allow the increased production PLP without over-expression of the biosynthetic pathway genes.

10

Ammonium Utilization

Tryptophanase reactions can be driven toward the synthetic direction (production of tryptophan from indole) by making ammonia more available or by removal of water. Reductive amination reactions, such as those catalyzed by glutamate dehydrogenase,

15   can also be driven forward by an excess of ammonium.

Ammonia can be made available as an ammonium carbonate or ammonium phosphate salt in a carbonate or phosphate buffered system. Ammonia can also be provided as ammonium pyruvate or ammonium formate. Alternatively, ammonia can be supplied

20   if the reaction is coupled with a reaction that generates ammonia, such as glutamate dehydrogenase or tryptophan dehydrogenase. Ammonia can be generated by addition of the natural substrates of EC 4.1.99.- (tyrosine or tryptophan), which will be hydrolyzed to phenol or indole, pyruvate and NH_3. This also allows for an increased yield of synthetic product over the normal equilibrium amount by allowing the

25   enzyme to hydrolyze its preferred substrate.

Removal of products and byproducts

The conversion of tryptophan to indole-3-pyruvate via a tryptophan aminotransferase can adversely affect the production rate of indole-3-pyruvate because the reaction

30   produces glutamate and requires the co-substrate 2-oxoglutarate (α-ketoglutarate). Glutamate can cause inhibition of the aminotransferase, and the reaction can consume large amounts of the co-substrate. Moreover, high glutamate concentrations can be detrimental to downstream separation processes.

The polypeptide glutamate dehydrogenase (GLDH) converts glutamate to 2-oxoglutarate, thereby recycling the co-substrate in the reaction catalyzed by tryptophan aminotransferase. GLDH also generates reducing equivalents (NADH or NADPH) that can be used to generate energy for the cell (ATP) under aerobic

5   conditions. The utilization of glutamate by GLDH also reduces byproduct formation. Additionally, the reaction generates ammonia, which can serve as a nitrogen source for the cell or as a substrate in a reductive amination for the final step shown in FIG. 1. Therefore, a production cell that over-expresses a GLDH polypeptide can be used to increase the yield and reduce the cost of media and/or separation processes.

10

In the tryptophan to monatin pathway, the amino donor of step three (e.g., glutamate or aspartate) can be converted back to the amino acceptor required for step 1 (e.g., 2-oxo-glutarate or oxaloacetate), if an aminotransferase from the appropriate enzyme classes is used. Utilization of two separate transaminases for this pathway, in which

15   the substrate of one transaminase does not competitively inhibit the activity of the other transaminase, can increase the efficiency of this pathway.

Many of the reactions in the described pathways are reversible and can, therefore, reach an equilibrium between substrates and products. The yield of the pathway can

20   be increased by continuous removal of the products from the polypeptides. For example, secretion of monatin into the fermentation broth using a permease or other transport protein, or selective crystallization of monatin from a biocatalytic reactor stream with concomitant recycle of substrates will increase the reaction yield.

25   Removal of byproducts via additional enzymatic reactions or via substitution of amino donor groups is another way to increase the reaction yield. Several examples are discussed in Example 13 and shown in FIGS. 11-13. For example, a byproduct can be produced that is unavailable to react in the reverse direction, either by phase change (evaporation) or by spontaneous conversion to an unreactive end product, such

30   as carbon dioxide.

Modulation of the Substrate Pools
The indole pool can be modulated by increasing production of tryptophan precursors and/or altering catabolic pathways involving indole-3-pyruvate and/or tryptophan.

For example, the production of indole-3-acetic acid from indole-3-pyruvate can be reduced or eliminated by functionally deleting the gene coding for EC 4.1.1.74 in the host cell. Production of indole from tryptophan can be reduced or eliminated by functionally deleting the gene coding for EC 4.1.99.1 in the host cell. Alternatively,

5   an excess of indole can be utilized as a substrate in an *in vitro* or *in vivo* process in combination with increased amounts of the gene coding for EC 4.1.99.1 (Kawasaki *et al., J. Ferm. and Bioeng.*, 82:604-6, 1996). In addition, genetic modifications can be made to increase the level of intermediates such as D-erythrose-4-phosphate and chorismate.

10

Tryptophan production is regulated in most organisms. One mechanism is via feedback inhibition of certain enzymes in the pathway; as tryptophan levels increase, the production rate of tryptophan decreases. Thus, when using a host cell engineered to produce monatin via a tryptophan intermediate, an organism can be used that is not

15   sensitive to tryptophan concentrations. For example, a strain of *Catharanthus roseus* that is resistant to growth inhibition by various tryptophan analogs was selected by repeated exposure to high concentrations of 5-methyltryptophan (Schallenberg and Berlin, Z Naturforsch 34:541-5, 1979). The resulting tryptophan synthase activity of the strain was less effected by product inhibition, likely due to mutations in the gene.

20   Similarly, a host cell used for monatin production can be optimized.

Tryptophan production can be optimized through the use of directed evolution to evolve polypeptides that are less sensitive to product inhibition. For example, screening can be performed on plates containing no tryptophan in the medium, but

25   with high levels of non-metabolizable tryptophan analogs. U.S. Pat. Nos. 5,756,345; 4,742,007; and 4,371,614 describe methods used to increase tryptophan productivity in a fermentation organism. The last step of tryptophan biosynthesis is the addition of serine to indole; therefore the availability of serine can be increased to increase tryptophan production.

30

The amount of monatin produced by a fermentation organism can be increased by increasing the amount of pyruvate produced by the host organism. Certain yeasts, such as *Trichosporon cutaneum* (Wang et al., Lett. Appl. Microbiol. 35:338-42, 2002) and *Torulopsis glabrata* (Li et al., Appl Microbiol. Biotechnol. 57:451-9, 2001)

28

overproduce pyruvate and can be used to practice the methods disclosed herein. In
addition, genetic modifications can be made to organisms to promote pyruvic acid
production, such as those in *E. coli* strain W1485*lip2* (Kawasaki et al., J. Ferm. and
Bioeng. 82:604-6, 1996).

5

Controlling Chirality

The taste profile of monatin can be altered by controlling its stereochemistry
(chirality). For example, different monatin isomers may be desired in different blends
of concentrations for different food systems. Chirality can be controlled via a

10   combination of pH and polypeptides.
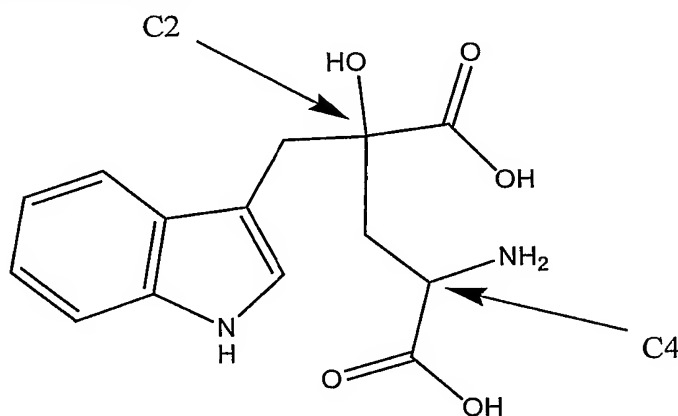
Racemization at the C-4 position of monatin (see numbered molecule above) can
occur by deprotonation and reprotonation of the alpha carbon, which can occur by a

15   shift in pH or by reaction with the cofactor PLP bound to an enzyme such as a
racemase or free in solution. In a microorganism, the pH is unlikely to shift enough to
cause the racemization, but PLP is abundant. Methods to control the chirality with
polypeptides depend upon the biosynthetic route utilized for monatin production.

20   When monatin is formed using the pathway shown in FIG. 2, the following can be
considered. In a biocatalytic reaction, the chirality of carbon-2 can be determined by
an enzyme that converts indole-3-pyruvate to MP. Multiple enzymes (e.g., from EC
4.1.2.-, 4.1.3.-) can convert indole-3-pyruvate to MP, thus, the enzyme that forms the
desired isomer can be chosen. Alternatively, the enantiospecificity of the enzyme that

25   converts indole-3-pyruvate to MP can be modified through the use of directed
evolution, or catalytic antibodies can be engineered to catalyze the desired reaction.

Once MP is produced (either enzymatically or by chemical condensation), the amino

group can be added stereospecifically using a transaminase, such as those described

herein. Either the R or S configuration of carbon-4 can be generated depending on

whether a D- or L- aromatic acid aminotransferase is used. Most aminotransferases

5   are specific for the L-isomer; however, D-tryptophan aminotransferases exist in

certain plants (Kohiba and Mito, Proceedings of the 8th International Symposium on

Vitamin B_6 and Carbonyl Catalysis, Osaka, Japan 1990). Moreover, D-alanine

aminotransferases (2.6.1.21), D-methionine-pyruvate aminotransferases (2.6.1.41),

and both (R)-3-amino-2-methylpropanoate aminotransferase (2.6.1.61) and (S)-3-

10   amino-2-methylpropanoate aminotransferase (2.6.1.22) have been identified. Certain

aminotransferases may only accept the substrate for this reaction with a particular

configuration at the C2 carbon. Therefore, even if the conversion to MP is not

stereospecific, the stereochemistry of the final product can be controlled through the

appropriate selection of a transaminase. Since the reactions are reversible, the

15   unreacted MP (undesired isomer) can be recycled back to its constituents, and a

racemic mixture of MP can be reformed.

Activating Substrates

Phosphorylated substrates, such as phosphoenolpyruvate (PEP), can be used in the

20   reactions disclosed herein. Phosphorylated substrates can be more energetically

favorable and, therefore, can be used to increase the reaction rates and/or yields. In

aldol condensations, the addition of a phosphate group stabilizes the enol tautomer of

the nucleophilic substrate, making it more reactive. In other reactions, a

phosphorylated substrate can provide a better leaving group. Similarly, substrates can

25   be activated by conversion to CoA derivatives or pyrophosphate derivatives.

Use of monatin in a sweetener composition

The S,S stereoisomer of monatin is approximately 50-200 times sweeter than sucrose

30   by weight. The R,R stereoisomer of monatin is approximately 2000-2400 times

sweeter than sucrose by weight. As discussed above, the sweetness of the monatin is

calculated using experienced sensory evaluators in a sweetness comparison procedure,

where a test sweetener solution is matched for sweetness intensity against one of a

series of reference solutions. The solutions may be prepared, for example, using a

buffer comprising 0.16% (v/w) citric acid and 0.02% (v/w) sodium citrate at ~pH 3.0. The intensity sweetness is calculated as the slope in a dose response curve, where the % sucrose is divided by the % monatin. *See e.g.,* FIG. 15 (R,R/S,S monatin dose response curve); FIG. 14 (R,R monatin dose response curve). The above-mentioned

5 monatin sweetness, as compared to sucrose sweetness, is obtained by measuring the slope at 8% sucrose equivalence values ("SEV").

Monatin is soluble in aqueous solutions in concentrations that are appropriate for consumption. Various blends of monatin stereoisomers may be qualitatively better in

10 certain matrices, or in blending with other sweeteners. Blends of monatin with other sweeteners may be used to maximize the sweetness intensity and/or profile, and minimize cost. Monatin may be used in combination with other sweeteners and/or other ingredients to generate a temporal profile similar to sucrose, or for other benefits.

15

For example, monatin may be blended with other nutritive and nonnutritive sweeteners to achieve particular flavor profiles or calorie targets. Thus, sweetener compositions can include combinations of monatin with one or more of the following sweetener types: (1) sugar alcohols (such as erythritol, sorbitol, maltitol, mannitol,

20 lactitol, xylitol, isomalt, low glycemic syrups, etc.); (2) other high intensity sweeteners (such as aspartame, sucralose, saccharin, acesulfame-K, stevioside, cyclamate, neotame, thaumatin, alitame, dihydrochalcone, monellin, glycyrrihizin, mogroside, phyllodulcin, mabinlin, brazzein, circulin, pentadin, etc.) and (3) nutritive sweeteners (such as sucrose, tagatose, invert sugar, fructose, corn syrup, high fructose

25 corn syrup (HFCS), glucose/dextrose, trehalose, isomaltulose, etc.). Monatin may be used in such blends as a taste modifier to suppress aftertaste, enhance other flavors such as lemon, or improve the temporal flavor profile. Data also indicate that monatin is quantitatively synergistic with cyclamates (which are used in Europe), but no significant quantitative synergy was noted with aspartame, saccharin, acesulfame-K,

30 sucralose, or carbohydrate sweeteners. Because monatin is not a carbohydrate, monatin can be used to partially or fully replace carbohydrate-containing sweeteners.

Monatin is stable in a dry form, and has a desirable taste profile alone or when mixed with carbohydrates. It does not appear to irreversibly break down, but rather forms

lactones and/or lactams at low pHs (in aqueous buffers) and reaches an equilibrium. It can racemize at the 4 position slowly over time in solution, but typically this occurs at high pHs. In general, the stability of monatin is comparable to or better than aspartame (Equal®) and the taste profile of monatin is comparable to or better than

5   other quality sweeteners, such aspartame (Equal®), alitame, and sucralose (Splenda®). Monatin does not have the undesirable aftertaste associated with some other high intensity sweeteners such as saccharin and stevioside.

Formulations of monatin sweeteners may be used as tabletop sugar substitutes.

10   Generally, when making tabletop sugar substitutes, one also employs bulking agents and/or carries to dilute the monatin and allow it to be easily measured.

In one embodiment, one may prepare convenient single-serving packets formulated to provide a sweetness comparable to that in 2 teaspoons (~8 grams) of granulated sugar.

15   Because S,S is 50-200 times sweeter than sucrose, 40-160 mg of S,S monatin delivers a sweetness comparable to that in 8 grams of granulated sugar. Thus, in one embodiment, allowing for +/– 25% sweetness optimization, single-serving packet 1 gram formulations of monatin may comprise approximately 40-200 mg of S,S monatin.

20

Likewise, because R,R is 2000-2400 times sweeter than sucrose, 3.3-4.0 mg of R,R monatin delivers a sweetness comparable to that in 8 grams of sugar. Thus, in another embodiment, allowing for +/– 25% sweetness optimization, single-serving packet 1 gram formulations of monatin may comprise approximately 3.3-5.0 mg of R,R

25   monatin. In another embodiment, packet formulations may comprise 40-200 mg of S,S monatin, 3.3-5.0 mg of R,R monatin or a combination thereof in the same or lesser amounts per gram total weight, to provide a sweetness comparable to that in 2 teaspoons of granulated sugar.

30   Tabletop sweetener packets often contain a total of 1 gram and a mixture of high-intensity sweetener and one or more bulking agents or carriers. A number of bulking agents or combinations of agents may be used in preparing the formulations. For example, in some embodiments, monatin can be spray dried with maltodextrin and/or

dextrose. Dextrose, for example, provides greater density and still allows for one packet (~1 g) of sweetener to round down to 0 Calories per serving.

In an embodiment, a formulation for a monatin-containing packet (1 g total) includes:

dextrose (nutritive) 0-99.7 wt %

maltodextrin 0-99.7 wt %

3.3-5.0 mg R,R monatin, 40-200 mg S,S monatin or a combination thereof in the same or lesser amounts.

In other embodiments, ready-to-use formulations of tabletop sweeteners are designed to have a sweetness and volume comparable to that of granulated sugar (sucrose). Such formulations can be used "spoon-for-spoon" in place of granulated sugar, which is especially useful in baking recipes. Compared to packet (~1 g) formulations, the ready-to-use "spoon-for-spoon" formulations generally contain more dilutant materials, such as bulking agents and/or carriers, which allow for common household measurements, such as by teaspoon, tablespoon or cup. These formulations typically have a less dense and less granular appearance than the packet versions. Thus, the ready-to-use "spoon-for-spoon" formulations may include different ingredients to reduce the density and allow the formulation to be used as an equal replacement for sugar.

For example, because dextrose is more dense, one may want to use maltodextrin alone when preparing the ready-to-use "spoon-for-spoon" formulations. The light density of maltodextrin allows the sweetener formulation to be used in equal volumes to sugar without accumulating the same (high) level of calories. This approach is especially useful when the monatin sweetener is used by the spoonful or cupful, such as in baking applications.

Because S,S is 50-200 times sweeter than sucrose, 5-20 mg of S,S monatin delivers a sweetness comparable to that in 1 gram of granulated sugar. Thus, in one embodiment, allowing for +/- 25% sweetness optimization, ready-to-use "spoon-for-spoon" formulations may comprise about 5-25 mg of S,S monatin/gram total weight. Likewise, because R,R is 2000-2400 times sweeter than sucrose, 0.4-0.5 mg of R,R monatin delivers a sweetness comparable to that in 1 gram of sugar. Thus, in another

embodiment, allowing for +/– 25% sweetness optimization, ready-to-use "spoon-for-spoon" formulations may comprise about 0.4-0.625 mg of R,R monatin. In another embodiment, the ready-to-use formulations may comprise 5-25 mg of S,S monatin, 0.4-0.625 mg of R,R monatin or a combination thereof in the same or lesser amounts

5   per gram total weight, to provide a sweetness comparable to that in granulated sugar.

In another embodiment, a formulation for a ready-to-use sweetener contains approximately 0.2-13 mg monatin per every half gram serving size, spray-dried onto approximately 500 mg maltodextrin.

10

In other embodiments, one can make monatin compositions in the form of cubes for use, for example, in restaurants. The cubes weigh approximately 8 grams and are of equivalent size to a standard cube of granulate sugar, which is 2.2 cm x 2.2 cm x 1 cm. These cube formulations contain monatin in amounts similar to those described

15   above for the ready-to-use formulations, so that the cubes contain a sweetness comparable to that in the standard sugar cube. Alternatively, one may also make ½ size cubes (4 grams, 1.1 cm x 2.2 cm x 1 cm). These formulations may be prepared to deliver a sweetness comparable to that in a standard sugar cube (and therefore contain approximately two times the sweetness of sucrose by weight).

20

Monatin tabletop formulations, such as those for packets or for the ready-to-use compositions, may also include other bulking agents or carriers. For instance, the formulations may include one or more fibers, such as inulin, arabinogalactan, hydrolyzed guar gum, polydextrose, microcrystalline cellulose, solka floc, starch,

25   modified starch, resistant starch, resistant maltodextrin, etc. Many fibers are highly soluble in water, have low viscosity, and bland flavor. Fibers are especially useful as a bulking agent because they are less caloric than other bulking agents.

For example, inulin delivers only 1.3 Calories per gram compared to 4 Calories per

30   gram for dextrose. De-sugared inulin, which contains <2% mono- and disaccharides delivers only 1.1 Calories per gram is a particularly good choice. Certain fibers, such as inulin, have the potential to impart a more granular sugar-like appearance to the formulation than maltodextrin, which is commonly used in cup-for-cup sugar substitutes. Fibers also provide the added benefits of fiber fortification, improved gut

health, improved calcium absorption, etc. Use of non-digestable carbohydrates, such as fibers or resistant starch derivatives, also results in less sugar content for those concerned with glycemic modulation (e.g., diabetics). For example, one may use resistant starch, resistant maltodextrin and inulin to obtain lower calorie, low

5   glycemic and low insulinogenic carriers or dilutants.

Likewise, monatin formulations may include one or more sugar alcohols, such as erythritol, sorbitol, xylitol, maltitol, mannitol and lactitol. In one embodiment, one may use erythritol in place of other carriers or bulking agents. Erythritol provides

10   certain advantages over dextrose or maltodextrin. For example, erythritol provides little to no calories, as compared to dextrose and maltodextrin—erythritol provides 0-0.2 Calories per gram, while dextrose provides 4 Calories per gram, and maltodextrin provides 2.8-3.2 Calories per gram. *See* ALTERNATIVE SWEETENERS, 3rd Ed., edited by Lyn O'Brien Nabors, Chapter 13 "Erythritol" by M.E. Embuscado and S. K. Patil

15   (Marcel Dekker, Inc, New York 2001). Also, when maltodextrin is sprayed dried, it looks like a powder rather than granular sugar. Erythritol, on the other hand, is monocrystalized and therefore looks like sugar. Thus, one can add monatin directly on top of erythritol.

20   In addition, the formulations may also include oligosaccharides, such as fructooligosaccharides, maltooligosaccharide, isomaltooligosaccharides, galatooligsaccharides, soybean oligosaccharides, and lactooligosaccharides. The formulations also may include sugars, such as sucrose, invert sugar, trehalose, isomerized sugar, glucose, fructose, lactose, malt sugar, D-xylose and isomerized

25   lactose, etc . If tablets are made, lactose can be added to increase bulk and shape the tablets.

The monatin and bulking agent combination may be produced by any means, including dry-mixed, co-spray dried, co-freeze dried, agglomerated, blended, co-

30   dried, extruded or other process.

One also may add flavorings and acidulants, such as cream of tartar, to the formulation mixtures for improved flavor, stability, and/or baking performance. One may also add flavor enhancers or stabilizers, such as Sucramask™.

The formulations may also include anti-caking or free-flow agents, such as silicon dioxide (silica), calcium silicate, magnesium silicate, calcium carbonate, sodium aluminosilicate, calcium aluminosilicate, potassium aluminosilicate, monocalcium

5   phosphate, dicalcium phosphate, tricalcium phosphate, talc, mannitol, etc.

In some embodiments, monatin is used in baking. In certain embodiments, honey or molasses is included in baking recipes to promote browning and good texture. For example, certain recipes may call for the addition of a tablespoon of molasses to

10   imitate the coloration due to normal browning of sugars.

In another embodiments, monatin is used to replace many of the polyols that are currently used in candies and other no sugar foods. These polyols have a laxation effect, and in the case of erythritol, also have a cooling effect. Erythritol has

15   substantially less laxation effect than other polyols. Other polyols have cooling effect as much as erythritol. Erythritol, as compared to traditional polyols, such as maltitol, contains fewer calories and a lower glycemic index. Monatin will even further reduce the caloric value and the glycemic index, without significantly increasing the cariogenicity. However, when replacing polyols or other traditional sweeteners (such

20   as sugars) with high intensity sweeteners, bulking agents are required to maintain both the volume and mouthfeel of the product. The most common bulking agents include inulin, maltodextrin, polydextrose, and sorbitol. Inulin can also improve uptake of dietary calcium by the body. Resistant starches and slowly digestible starches can also be utilized as bulking agents or diluents of high intensity sweeteners. Monatin

25   can be used in combination with a number of sweeteners, carbohydrates, and bulking agents.

It is expected that monatin tabletop blends, as compared to other sweeteners, will have a longer shelf-life, greater heat and acid stability, as well as better taste

30   characteristics and marketing advantages.

EXAMPLES

EXAMPLE 1

Cloning and Expression of Tryptophan Aminotransferases

This example describes methods that were used to clone tryptophan aminotransferases, which can be used to convert tryptophan to indole-3-pyruvate.

5

Experimental Overview

Eleven genes encoding aminotransferases were cloned into *E. coli*. These genes were *Bacillus subtilis* D-alanine aminotransferase (*dat*, Genbank Accession No. Y14082.1 bp 28622-29470 and Genbank Accession No. NP_388848.1, nucleic acid sequence

10   and amino acid sequence, respectively), *Sinorhizobium meliloti* (also termed *Rhizobium meliloti*) tyrosine aminotransferase (*tatA*, SEQ ID NOS: 1 and 2, nucleic acid sequence and amino acid sequence, respectively), *Rhodobacter sphaeroides* strain 2.4.1 tyrosine aminotransferase (*tatA* asserted by homology, SEQ ID NOS: 3 and 4, nucleic acid sequence and amino acid sequence, respectively), *R. sphaeroides*

15   35053 tyrosine aminotransferase (asserted by homology, SEQ ID NOS: 5 and 6, nucleic acid sequence and amino acid sequence, respectively), *Leishmania major* broad substrate aminotransferase (*bsat*, asserted by homology to peptide fragments from *L. mexicana*, SEQ ID NOS: 7 and 8, nucleic acid sequence and amino acid sequence, respectively), *Bacillus subtilis* aromatic aminotransferase (*araT*, asserted by

20   homology, SEQ ID NOS: 9 and 10, nucleic acid sequence and amino acid sequence, respectively), *Lactobacillus amylovorus* aromatic aminotransferase (*araT* asserted by homology, SEQ ID NOS: 11 and 12, nucleic acid sequence and amino acid sequence, respectively), *R. sphaeroides* 35053 multiple substrate aminotransferase (asserted by homology, SEQ ID NOS: 13 and 14, nucleic acid sequence and amino acid sequence,

25   respectively), *Rhodobacter sphaeroides* strain 2.4.1 multiple substrate aminotransferase (*msa* asserted by homology, Genbank Accession No. AAAE01000093.1, bp 14743-16155 and Genbank Accession No. ZP00005082.1, nucleic acid sequence and amino acid sequence, respectively), *Escherichia coli* aspartate aminotransferase (*aspC*, Genbank Accession No. AE000195.1 bp 2755-

30   1565 and Genbank Accession No. AAC74014.1, nucleic acid sequence and amino acid sequence, respectively), and *E. coli* tyrosine aminotransferase (*tyrB*, SEQ ID NOS: 31 and 32, nucleic acid sequence and amino acid sequence, respectively).

The genes were cloned, expressed, and tested for activity in conversion of tryptophan to indole-3-pyruvate, along with commercially available enzymes. All eleven clones had activity.

5   *Identification of Bacterial Strains that Can Contain Polypeptides with the Desired Activity*

No genes in the NCBI (National Center for Biotechnology Information) database were designated as tryptophan aminotransferases. However, organisms having this enzymatic activity have been identified. L-tryptophan aminotransferase (TAT)

10   activity has been measured in cell extracts or from purified protein from the following sources: Rhizobacterial isolate from *Festuca octoflora*, pea mitochondria and cytosol, sunflower crown gall cells, *Rhizobium leguminosarum* biovar *trifoli*, *Erwinia herbicola* pv gypsophilae, *Pseudomonas syringae* pv. savastanoi, *Agrobacterium tumefaciens*, *Azospirillum lipferum* & *brasilense*, *Enterobacter cloacae*, *Enterobacter*

15   *agglomerans*, *Bradyrhizobium elkanii*, *Candida maltosa*, *Azotobacter vinelandii*, rat brain, rat liver, *Sinorhizobium meliloti*, *Pseudomonas fluorescens* CHA0, *Lactococcus lactis*, *Lactobacillus casei*, *Lactobacillus helveticus*, wheat seedlings, barley, *Phaseolus aureus* (mung bean), *Saccharomyces uvarum* (carlsbergensis), *Leishmania* sp., maize, tomato shoots, pea plants, tobacco, pig, *Clostridium*

20   *sporogenes*, and *Streptomyces griseus*.

EXAMPLE 2

Conversion of Indole-3-lactate to Indole-3-pyruvate

As shown in FIGS. 1 and 3, indole-3-lactic acid can be used to produce indole-3-

25   pyruvate. Conversion between lactic acid and pyruvate is a reversible reaction, as is conversion between indole-3-pyruvate and indole-3-lactate. The oxidation of indole-lactate was typically followed due to the high amount of background at 340 nm from indole-3-pyruvate.

30   The standard assay mixture contained 100 mM potassium phosphate, pH 8.0, 0.3 mM NAD^+, 7 units of lactate dehydrogenase (LDH) (Sigma-L2395, St. Louis, MO), and 2 mM substrate in 0.1 mL. The assay was performed in duplicate in a UV-transparent microtiter plate, using a Molecular Devices SpectraMax Plus platereader. Polypeptide and buffer were mixed and pipetted into wells containing the indole-3-lactic acid and

NAD^+ and the absorbance at 340 nm of each well was read at intervals of 9 seconds after brief mixing. The reaction was held at 25°C for 5 minutes. The increase in absorbance at 340 nm follows the production of NADH from NAD^+. Separate negative controls were performed without NAD^+ and without substrate. D-LDH from

5 *Leuconostoc mesenteroides* (Sigma catalog number L2395) appeared to exhibit more activity with the indole-derivative substrates than did L-LDH from *Bacillus stearothermophilus* (Sigma catalog number L5275).

Similar methods were utilized with D-lactic acid and NAD+ or NADH and pyruvate,

10 the natural substrates of D-LDH polypeptides. The V_{max} for the reduction of pyruvate was 100-1000 fold higher than the V_{max} for the oxidation of lactate. The V_{max} for the oxidation reaction of indole-3-lactic with D-LDH was approximately one-fifth of that with lactic acid. The presence of indole-3-pyruvate was also measured by following the change in absorbance at 327 (the enol-borate derivative) using 50 mM sodium

15 borate buffer containing 0.5 mM EDTA and 0.5 mM sodium arsenate. Small, but repeatable, absorbance changes were observed, as compared to the negative controls for both L and D-LDH polypeptides.

Additionally, broad specificity lactate dehydrogenases (enzymes with activity

20 associated with EC 1.1.1.27, EC 1.1.1.28, and/or EC 1.1.2.3) can be cloned and used to make indole-3-pyruvate from indole-3-lactic acid. Sources of broad specificity dehydrogenases include *E. coli, Neisseria gonorrhoeae*, and *Lactobacillus plantarum*.

Alternatively, indole-3-pyruvate can be produced by contacting indole-3-lactate with

25 cellular extracts from *Clostridium sporogenes* which contain an indolelactate dehydrogenase (EC 1.1.1.110); or *Trypanosoma cruzi epimastigotes* cellular extracts which contain *p*-hydroxyphenylactate dehydrogenase (EC 1.1.1.222) known to have activity on indole-3-pyruvate; or *Pseudomonas acidovorans* or *E. coli* cellular extracts, which contain an imidazol-5-yl lactate dehydrogenase (EC 1.1.1.111); or

30 *Coleus blumei*, which contains a hydroxyphenylpyruvate reductase (EC 1.1.1.237); or *Candida maltosa* which contains a D-aromatic lactate dehydrogenase (EC 1.1.1.222). References describing such activities include, Nowicki *et al.* (*FEMS Microbiol Lett* 71:119-24, 1992), Jean and DeMoss (*Canadian J. Microbiol.* 14 1968, Coote and Hassall (*Biochem. J.* 111: 237-9, 1969), Cortese *et al.* (*C.R. Seances Soc. Biol. Fil.*

162 390-5, 1968), Petersen and Alfermann (*Z. Naturforsch. C: Biosci.* 43 501-4,

1988), and Bhatnagar *et al.* (*J. Gen Microbiol* 135:353-60, 1989). In addition, a

lactate oxidase such as the one from *Pseudomonas sp.* (Gu *et al.* J. Mol. Catalysis B:

Enzymatic: 18:299-305, 2002), can be utilized for oxidation of indole-3-lactic to

5   indole-3-pyruvate.

EXAMPLE 3

Conversion of L-tryptophan to Indole-3-pyruvate utilizing L-amino acid oxidase

This example describes methods used to convert tryptophan to indole-3-pyruvate via

10   an oxidase (EC 1.4.3.2), as an alternative to using a tryptophan aminotransferase as

described in Example 1. L-amino acid oxidase was purified from *Crotalus durissus*

(Sigma, St. Louis, MO, catalog number A-2805). The accession numbers of L-amino

acid oxidases for molecular cloning include: CAD21325.1, AAL14831, NP_490275,

BAB78253, A38314, CAB71136, JE0266, T08202, S48644, CAC00499, P56742,

15   P81383, O93364, P81382, P81375, S62692, P23623, AAD45200, AAC32267,

CAA88452, AP003600, and Z48565.

Reactions were performed in microcentrifuge tubes in a total volume of 1 mL,

incubated for 10 minutes while shaking at 37°C. The reaction mix contained 5 mM L-

20   tryptophan, 100 mM sodium phosphate buffer pH 6.6, 0.5 mM sodium arsenate, 0.5

mM EDTA, 25 mM sodium tetraborate, 0.016 mg catalase (83 U, Sigma C-3515),

0.008 mg FAD (Sigma), and 0.005-0.125 Units of L-amino acid oxidase. Negative

controls contained all components except tryptophan, and blanks contained all

components except the oxidase. Catalase was used to remove the hydrogen peroxide

25   formed during the oxidative deamination. The sodium tetraborate and arsenate were

used to stabilize the enol-borate form of indole-3-pyruvate, which shows a maximum

absorbance at 327 nm. Indole-3-pyruvate standards were prepared at concentrations

of 0.1-1 mM in the reaction mix.

The purchased L-amino acid oxidase had a specific activity of 540 μg indole-3-

30   pyruvate formed per minute per mg protein. This is the same order of magnitude as

the specific activity of tryptophan aminotransferase enzymes.

EXAMPLE 4

Converting Indole-3-pyruvate to 2-hydroxy 2-(indol-3-ylmethyl)-4-keto glutaric acid
with an Aldolase

This example describes methods that can be used to convert indole-3-pyruvate to MP

5   using an aldolase (lyase) (FIG. 2). Aldol condensations are reactions that form
carbon-carbon bonds between the β-carbon of an aldehyde or ketone and the carbonyl
carbon of another aldehyde or ketone. A carbanion is formed on the carbon adjacent
to the carbonyl group of one substrate, and serves as a nucleophile attacking the
carbonyl carbon of the second substrate (the electrophilic carbon). Most commonly,

10   the electrophilic substrate is an aldehyde, so most aldolases fall into the EC 4.1.2.-
category. Quite often, the nucleophilic substrate is pyruvate. It is less common for
aldolases to catalyze the condensation between two keto-acids or two aldehydes.

However, aldolases that catalyze the condensation of two carboxylic acids have been

15   identified. For example, EP 1045-029 describes the production of L-4-hydroxy-2-
ketoglutaric acid from glyoxylic acid and pyruvate using a *Pseudomonas* culture (EC
4.1.3.16). In addition, 4-hydroxy-4-methyl-2-oxoglutarate aldolase (4-hydroxy-4-
methyl-2-oxoglutarate pyruvate lyase, EC 4.1.3.17) can catalyze the condensation of
two keto acids. Therefore, similar aldolase polypeptides were used to catalyze the

20   condensation of indole-3-pyruvate with pyruvate.

Cloning

4-Hydroxy-4-methyl-2-oxoglutarate pyruvate lyases (ProA aldolase, EC 4.1.3.17) and
4-hydroxy-2-oxoglutarate glyoxylate-lyase (KHG aldolase, EC 4.1.3.16) catalyze

25   reactions very similar to the aldolase reaction of FIG. 2. Primers were designed with
compatible overhangs for the pET30 Xa/LIC vector (Novagen, Madison, WI).

Activity Results with proA *gene products*

Both the *C. testosteroni proA* and *S. meliloti SMc00502* gene constructs had high

30   levels of expression when induced with IPTG. The recombinant proteins were highly
soluble, as determined by SDS-PAGE analysis of total protein and cellular extract
samples. The *C. testosteroni* gene product was purified to > 95% purity. Because the
yield of the *S. meliloti* gene product was very low after affinity purification using a
His-Bind cartridge, cellular extract was used for the enzymatic assays.

Both recombinant aldolases catalyzed the formation of MP from indole-3-pyruvate and pyruvate. The presence of both divalent magnesium and potassium phosphate were required for enzymatic activity. No product was apparent when indole-3-

5   pyruvate, pyruvate, or potassium phosphate was absent. A small amount of the product was also formed in the absence of enzyme (typically one order of magnitude less than when enzyme was present).

The product peak eluted from the reverse phase C18 column slightly later than the

10   indole-3-pyruvate standard, the mass spectrum of this peak showed a collisionally-induced parent ion ($[M + H]+$) of 292.1, the parent ion expected for the product MP. The major daughter fragments present in the mass spectrum included those with m/z =158 (1H-indole-3-carbaldehyde carbonium ion), 168 (3-buta-1,3-dienyl-1H-indole carbonium ion), 274 (292 - H_2O), 256 (292 - 2 H_2O), 238 (292 - 3 H2O), 228 (292 -

15   CH4O3), and 204 (loss of pyruvate). The product also exhibited a UV spectrum characteristic of other indole-containing compounds such as tryptophan, with the λ_{max} of 279-280 and a small shoulder at approximately 290 nm.

The amount of MP produced by the *C. testosteroni* aldolase increased with an

20   increase in reaction temperature from room temperature to 37°C, amount of substrate, and amount of magnesium. The synthetic activity of the enzyme decreased with increasing pH, the maximum product observed was at pH 7. Based on tryptophan standards, the amount of MP produced under a standard assay using 20 μg of purified protein was approximately 10-40 μg per one mL reaction.

25

Due to the high degree of homology of the *S. meliloti* and *C. testosteroni* ProA aldolase coding sequences with the other genes described above, it is expected that all of the recombinant gene products can catalyze this reaction. Moreover, it is expected that aldolases that have threonine (T) at positions 59 and 87, arginine (R) at 119,

30   aspartate (D) at 120, and histidine (H) at 31 and 71, (based on the numbering system of *C. testosteroni*) will have similar activity.

Activity Results with khg *gene products*

Both the *B. subtilis* and *E. coli khg* gene constructs had high levels of expression of protein when induced with IPTG, while the *S. meliloti khg* had a lower level of expression. The recombinant proteins were highly soluble, as judged by SDS-PAGE analysis of total proteins and cellular extracts. The *B. subtilis* and *E. coli khg* gene

5   products were purified to > 95% purity; the yield of the *S. meliloti* gene product was not as high after affinity purification using a His-Bind cartridge.

There is no evidence that magnesium and phosphate are required for activity for this enzyme. However, the literature reports performing the assays in sodium phosphate

10   buffer, and the enzyme reportedly is bifunctional and has activity on phosphorylated substrates such as 2-keto-3-deoxy-6-phosphogluconate (KDPG). The enzymatic assays were performed as described above, and in some instances the phosphate was omitted. The results indicate that the recombinant KHG aldolases produced MP, but were not as active as the ProA aldolases. In some cases the level of MP produced by

15   KHG was almost identical to the amount produced by magnesium and phosphate alone. Phosphate did not appear to increase the KHG activities. The *Bacillus* enzyme had the highest activity, approximately 20-25% higher activity than the magnesium and phosphate alone, as determined by SRM (see Example 10). The *Sinorhizobium* enzyme had the least amount of activity, which can be associated with folding and

20   solubility problems noted in the expression. All three enzymes have the active site glutamate (position 43 in *B. subtilis* numbering system) as well as the lysine required for Shiff base formation with pyruvate (position 130); however, the *B. subtilis* enzyme contains a threonine in position 47, an active site residue, rather than arginine. The *B. subtilis* KHG is smaller and appears to be in a cluster distinct from the *S. meliloti* and

25   *E. coli* enzymes, with other enzymes having the active site threonine. The differences in the active site may be the reason for the increased activity of the *B. subtilis* enzyme.

Improvement of Aldolase Activity

Catalytic antibodies can be as efficient as natural aldolases, accept a broad range of

30   substrates, and can be used to catalyze the reaction shown in FIG. 2.

Aldolases can also be improved by directed evolution, for example as previously described for a KDPG aldolase (highly homologous to KHG described above) evolved by DNA shuffling and error-prone PCR to remove the requirement for

phosphate and to invert the enantioselectivity. The KDPG aldolase polypeptides are useful in biochemical reactions since they are highly specific for the donor substrate (herein, pyruvate), but are relatively flexible with respect to the acceptor substrate (i.e. indole-3-pyruvate) (Koeller & Wong, *Nature* 409:232-9, 2001). KHG aldolase has

5   activity for condensation of pyruvate with a number of carboxylic acids. Mammalian versions of the KHG aldolase are thought to have broader specificity than bacterial versions, including higher activity on 4-hydroxy 4-methyl 2-oxoglutarate and acceptance of both stereoisomers of 4-hydroxy-2-ketoglutarate. Bacterial sources appear to have a 10-fold preference for the R isomer. There are nearly 100 KHG

10   homologs available in genomic databases, and activity has been demonstrated in *Pseudomonas, Paracoccus, Providencia, Sinorhizobium, Morganella, E. coli,* and mammalian tissues. These enzymes can be used as a starting point for tailoring the enantiospecificity that is desired for monatin production.

15   Aldolases that utilize pyruvate and another substrate that is either a keto acid and/or has a bulky hydrophobic group like indole can be "evolved" to tailor the polypeptide's specificity, speed, and selectivity. In addition to KHG and ProA aldolases demonstrated herein, examples of these enzymes include, but are not limited to: KDPG aldolase and related polypeptides (KDPH); transcarboxybenzalpyruvate

20   hydratase-aldolase from *Nocardioides* st; 4-(2-carboxyphenyl)-2-oxobut-3-enoate aldolase (2'-carboxybenzalpyruvate aldolase) which condenses pyruvate and 2-carboxybenzaldehyde (an aromatic ring-containing substrate); trans-O-hydroxybenzylidenepyruvate hydratase-aldolase from *Pseudomonas putida* and *Sphingomonas aromaticivorans*, which also utilizes pyruvate and an aromatic-

25   containing aldehyde as substrates; 3-hydroxyaspartate aldolase (erythro-3-hydroxy-L-aspartate glyoxylate lyase), which uses 2-oxo acids as the substrates and is thought to be in the organism *Micrococcus denitrificans*; benzoin aldolase (benzaldehyde lyase), which utilizes substrates containing benzyl groups; dihydroneopterin aldolase; L-threo-3-phenylserine benzaldehyde-lyase (phenylserine aldolase) which condenses

30   glycine with benzaldehyde; 4-hydroxy-2-oxovalerate aldolase; 1,2-dihydroxybenzylpyruvate aldolase; and 2-hydroxybenzalpyruvate aldolase.

A polypeptide having the desired activity can be selected by screening clones of interest using the following methods. Tryptophan auxotrophs are transformed with

44

vectors carrying the clones of interest on an expression cassette and are grown on a medium containing small amounts of monatin or MP. Since aminotransferases and aldolase reactions are reversible, the cells are able to produce tryptophan from a racemic mixture of monatin. Similarly, organisms (both recombinant and wildtype)

5   can be screened by ability to utilize MP or monatin as a carbon and energy source. One source of target aldolases is expression libraries of various *Pseudomonas* and rhizobacterial strains. Pseudomonads have many unusual catabolic pathways for degradation of aromatic molecules and they also contain many aldolases; whereas the rhizobacteria contain aldolases, are known to grow in the plant rhizosphere, and have

10   many of the genes described for construction of a biosynthetic pathway for monatin.

EXAMPLE 5

Chemical Synthesis of the Monatin Precursor

Example 4 described a method of using an aldolase to convert indole-3-pyruvate to

15   MP. This example describes an alternative method of chemically synthesizing MP. MP can be formed using a typical aldol-type condensation (FIG. 4). Briefly, a typical aldol-type reaction involves the generation of a carbanion of the pyruvate ester using a strong base, such as LDA (lithium diisopropylamide), lithium hexamethyldisilazane or butyl lithium. The carbanion that is generated reacts with the indole-pyruvate to

20   form the coupled product.

Protecting groups that can be used for protecting the indole nitrogen include, but are not limited to: t-butyloxycarbonyl (Boc), and benzyloxycarbonyl (Cbz). Blocking groups for carboxylic acids include, but are not limited to, alkyl esters (for example,

25   methyl, ethyl, benzyl esters). When such protecting groups are used, it is not possible to control the stereochemistry of the product that is formed. However, if R2 and/or R3 are chiral protecting groups (FIG. 4), such as (S)-2-butanol, menthol, or a chiral amine, this can favor the formation of one MP enantiomer over the other.

30   ## EXAMPLE 6

Conversion of Tryptophan or Indole-3-Pyruvate to Monatin

An *in vitro* process utilizing two enzymes, an aminotransferase and an aldolase, produced monatin from tryptophan and pyruvate. In the first step alpha-ketoglutarate was the acceptor of the amino group from tryptophan in a transamination reaction

generating indole-3-pyruvate and glutamate. An aldolase catalyzed the second reaction in which pyruvate was reacted with indole-3-pyruvate, in the presence of Mg^{2+} and phosphate, generating the alpha-keto derivative of monatin (MP), 2-hydroxy-2-(indol-3-ylmethyl)-4-ketoglutaric acid. Transfer of the amino group from

5   the glutamate formed in the first reaction produced the desired product, monatin. Purification and characterization of the product established that the isomer formed was S,S-monatin. Alternative substrates, enzymes, and conditions are described as well as improvements that were made to this process.

10   *Enzymes*

The aldolase, 4-hydroxy-4-methyl-2-oxoglutarate pyruvate lyase (ProA aldolase, *proA* gene) (EC 4.1.3.17) from *Comamonas testosteroni* was cloned, expressed and purified as described in Example 4. The 4-hydroxy-2-oxoglutarate glyoxylate lyases (KHG aldolases) (EC 4.1.3.16) from *B. subtilis*, *E. coli*, and *S. meliloti* were cloned,

15   expressed and purified as described in Example 4.

The aminotransferases used in conjunction with the aldolases to produce monatin were L-aspartate aminotransferase encoded by the *E. coli aspC* gene, the tyrosine aminotransferase encoded by the *E. coli tyrB* gene, the *S. meliloti* TatA enzyme, the

20   broad substrate aminotransferase encoded by the *L. major bsat* gene, or the glutamic-oxaloacetic transaminase from pig heart (Type IIa). The cloning, expression and purification of the non-mammalian proteins are described in Example 1. Glutamic-oxaloacetic transaminase from pig heart (type IIa) was obtained from Sigma (# G7005).

25

Method using ProA aldolase and L-aspartate aminotransferase

The reaction mixture contained 50 mM ammonium acetate, pH 8.0, 4 mM $MgCl_2$, 3 mM potassium phosphate, 0.05 mM pyridoxal phosphate, 100 mM ammonium pyruvate, 50 mM tryptophan, 10 mM alpha-ketoglutarate, 160 mg of recombinant *C.*

30   *testosteroni* ProA aldolase (unpurified cell extract, ~30% aldolase), 233 mg of recombinant *E. coli* L-aspartate aminotransferase (unpurified cell extract, ~40% aminotransferase) in one liter. All components except the enzymes were mixed together and incubated at 30°C until the tryptophan dissolved. The enzymes were

then added and the reaction solution was incubated at 30°C with gentle shaking (100 rpm) for 3.5 hours. At 0.5 and 1 hour after the addition of the enzymes aliquots of solid tryptophan (50 mmoles each) were added to the reaction. All of the added tryptophan did not dissolve, but the concentration was maintained at 50 mM or

5   higher. After 3.5 hours, the solid tryptophan was filtered off. Analysis of the reaction mixture by LC/MS using a defined amount of tryptophan as a standard showed that the concentration of tryptophan in the solution was 60.5 mM and the concentration of monatin was 5.81 mM (1.05 g).

10   The following methods were used to purify the final product. Ninety percent of the clear solution was applied to a column of BioRad AG50W-X8 resin (225 mL; binding capacity of 1.7 meq/mL). The column was washed with water, collecting 300 mL fractions, until the absorbance at 280 nm was <5% of the first flow through fraction. The column was then eluted with 1 M ammonium acetate, pH 8.4, collecting 4 300-

15   mL fractions. All 4 fractions contained monatin and were evaporated to 105 mL using a roto-evaporator with a tepid water bath. A precipitate formed as the volume reduced and was filtered off over the course of the evaporation process.

Analysis of the column fractions by LC/MS showed that 99% of the tryptophan and

20   monatin bound to the column. The precipitate that formed during the evaporation process contained >97% tryptophan and <2% of monatin. The ratio of tryptophan to product in the supernatant was approximately 2:1.

The supernatant (7 ml) was applied to a 100 mL Fast Flow DEAE Sepharose

25   (Amersham Biosciences) column previously converted to the acetate form by washing with 0.5 L 1 M NaOH, 0.2 L water, 1.0 L of 1.0 M ammonium acetate, pH 8.4, and 0.5 L water. The supernatant was loaded at <2 mL/min and the column was washed with water at 3-4 mL/min until the absorbance at 280 nm was ~0. Monatin was eluted with 100 mM ammonium acetate; pH 8.4, collecting 4 100-mL fractions.

30

Analysis of the fractions showed that the ratio of tryptophan to monatin in the flow through fractions was 85:15 and the ratio in the eluent fractions was 7:93. Assuming the extinction coefficient at 280 nm of monatin is the same as tryptophan, the eluent

fractions contained 0.146 mmole of product. Extrapolation to the total 1 L reaction would produce ~2.4 mmoles (~710 mg) of monatin, for a recovery of 68%.

5   The eluent fractions from the DEAE Sepharose column were evaporated to <20 mL. An aliquot of the product was further purified by application to a C_8 preparative reversed-phase column using the same chromatographic conditions as those described in Example 10 for the analytical-scale monatin characterization. Waters Fractionlynx™ software was employed to trigger automated fraction collection of monatin based on detection of the $m/z = 293$ ion. The fraction from the C_8 column

10   with the corresponding protonated molecular ion for monatin was collected, evaporated to dryness, and then dissolved in a small volume of water. This fraction was used for characterization of the product.

The resulting product was characterized using the following methods.

15   **UV/Visible Spectroscopy.** UV/visible spectroscopic measurements of monatin produced enzymatically were carried out using a Cary 100 Bio UV/visible spectrophotometer. The purified product, dissolved in water, showed an absorption maximum of 280 nm with a shoulder at 288 nm, characteristics typical of indole containing compounds.

20

LC/MS Analysis. Analyses of mixtures for monatin derived from the *in vitro* biochemical reactions were carried out as described in Example 10. A typical LC/MS analysis of monatin in an *in vitro* enzymatic synthetic mixture is illustrated in FIG. 5. The lower panel of FIG. 5 illustrates a selected ion chromatogram for the protonated

25   molecular ion of monatin at $m/z = 293$. This identification of monatin in the mixture was corroborated by the mass spectrum illustrated in FIG. 6. Analysis of the purified product by LC/MS showed a single peak with a molecular ion of 293 and absorbance at 280 nm. The mass spectrum was identical to that shown in FIG. 6.

30   **MS/MS Analysis.** LC/MS/MS daughter ion experiments, as described in Example 10, were also performed on monatin. A daughter ion mass spectrum of monatin is illustrated in FIG. 7. Tentative structural assignments of all fragment ions labeled in FIG. 7 were made. These include fragment ions of $m/z = 275$ (293 − H_2O), 257 (293-

(2 x H$_2$O)), 230 (275-COOH), 212 (257-COOH), 168 (3-buta-1,3-dienyl-1H-indole carbonium ion), 158 (1H-indole-3-carbaldehyde carbonium ion), 144 (3-ethyl-1H-indole carbonium ion), 130 (3-methylene-1H-indole carbonium ion), and 118 (indole carbonium ion). Many of these are the same as those obtained for MP (Example 4),

5   as expected if derived from the indole portion of the molecule. Some are 1 mass unit higher than those seen for MP, due to the presence of an amino group instead of a ketone.

Accurate Mass Measurement of Monatin. FIG. 8 illustrates the mass spectrum
10   obtained for purified monatin employing an Applied Biosystems-Perkin Elmer Q-Star hybrid quadrupole/time-of-flight mass spectrometer. The measured mass for protonated monatin using tryptophan as an internal mass calibration standard was 293.1144. The calculated mass of protonated monatin, based on the elemental composition C$_{14}$H$_{17}$N$_2$O$_5$ is 293.1137. This is a mass measurement error of less than
15   2 parts per million (ppm), providing conclusive evidence of the elemental composition of monatin produced enzymatically.

NMR Spectroscopy. The NMR experiments were performed on a Varian Inova 500 MHz instrument. The sample of monatin (~3 mg) was dissolved in 0.5 ml of D$_2$O.
20   Initially, the solvent (D$_2$O) was used as the internal reference at 4.78 ppm. Since the peak for water was large, the ^{1}H-NMR was run with suppression of the peak for water. Subsequently, due to the broadness of the water peak, the C-2 proton of monatin was used as the reference peak, and set at the published value of 7.192 ppm.

25   For ^{13}C-NMR, an initial run of several hundred scans indicated that the sample was too dilute to obtain an adequate ^{13}C spectrum in the allotted time. Therefore, a heteronuclear multiple quantum coherence (HMQC) experiment was performed, which enabled the correlation of the hydrogens and the carbons to which they were attached, and also providing information on the chemical shifts of the carbons.
30

A summary of the ^{1}H and HMQC data is shown in Tables 2 and 3. By comparison to published values, the NMR data indicated that the enzymatically produced monatin was either (S,S), (R,R), or a mixture of both.

Chiral LC/MS Analysis. To establish that the monatin produced *in vitro* was one isomer, and not a mixture of the (R,R) and (S,S) enantiomers, chiral LC/MS analyses were carried out using the instrumentation described in Example 10.

5   Chiral LC separations were made using an Chirobiotic T (Advanced Separations Technology) chiral chromatography column at room temperature. Separation and detection, based on published protocols from the vendor, were optimized for the R-(D) and S- (L) isomers of tryptophan. The LC mobile phase consisted of A) water containing 0.05% (v/v) trifluoroacetic acid; B) Methanol containing 0.05% (v/v)

10   trifluoroacetic acid. The elution was isocratic at 70% A and 30% B. The flow rate was 1.0 mL/min, and PDA absorbance was monitored from 200 nm to 400 nm. The instrumental parameters used for chiral LC/MS analysis of tryptophan and monatin are identical to those described in Example 10 for LC/MS analysis. Collection of mass spectra for the region *m/z* 150–400 was utilized. Selected ion chromatograms

15   for protonated molecular ions ($[M + H]^+$ = 205 for both R- and S-tryptophan and $[M + H]^+$ = 293 for monatin) allowed direct identification of these analytes in the mixtures.

The chromatograms of R- and S-tryptophan and monatin, separated by chiral chromatography and monitored by MS, are shown in FIG. 9. The single peak in the

20   chromatogram of monatin indicates that the compound is one isomer, with a retention time almost identical to S-tryptophan.

TABLE 2

^{1}H NMR data
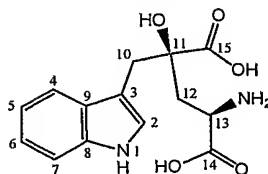
	Cargill		Vleggaar et al.[1]		Takeshi et al.[2]	
Atom	δ_H	**J(HH) Hz**	δ_H	**J(HH) Hz**	δ_H	**J(HH) Hz**
2	7.192 (1H, s)		7.192 (s)		7.18 (s)	
4	7.671 (d)	7.99	7.686 (d)	7.9	7.67 (d)	8.0
5	7.104 (dd)	7.99	7.102 (dd)	8.0, 8.0	7.11 (dd)	7.5, 7.5
6	7.178 (dd)	*	7.176 (dd)	8.0, 8.0	7.17 (dd)	7.5, 7.5
7	7.439 (d)	7.99	7.439 (d)	8.1	7.43 (d)	8.0
10a	3.242(d)	14.5	3.243 (d)	14.3	3.24 (d)	14.5
10b	3.033 (d)	14.5	3.051 (d)	14.3	3.05 (d)	14.5
12	2.626 (dd) 2.015 (dd)	15.5, 1.5 15.0, 12.0	2.651 (dd) 2.006 (dd)	15.3, 1.7 15.3, 11.7	2.62 (dd) 2.01 (dd)	15.5, 1.8 15.5, 12.0
13	3.571 (dd)	10.75*, 1.5	3.168 (dd)	11.6, 1.8	3.57 (dd)	12.0, 1.8

[1] Vleggaar *et al.* (*J.C.S. Perkin Trans.* 1:3095-8, 1992).
[2] Takeshi and Shusuke (JP2002060382, 2002-02-26).

TABLE 3
^{13}C NMR data (from HMQC spectrum)

	Cargill	Vleggaar et al.[1]
Atom	δ_C	δ_C
2	126.1	126.03
3	*	110.31
4	120.4	120.46
5	120.2	120.25
6	122.8	122.74
7	112.8	112.79
8	*	137.06
9	*	129.23
10a	36.4	36.53
12	39.5	39.31
13	54.9	54.89
14	*	175.30
15	*	181.18

[1] Vleggaar *et al.* (*J.C.S. Perkin Trans.* 1:3095-8, 1992).

Polarimetry. The optical rotation was measured on a Rudolph Autopol III polarimeter. The monatin was prepared as a 14.6 mg/mL solution in water. The expected specific rotation ($[\alpha]_D^{20}$) for S,S monatin (salt form) is -49.6 for a 1 g/mL solution in water (Vleggaar *et al*). The observed $[\alpha]_D^{20}$ was -28.1 for the purified,

5   enzymatically produced monatin indicating that it was the S, S isomer.

Improvements

The reaction conditions, including reagent and enzyme concentrations, were optimized and yields of 5-10 mg/mL were produced using the following reagent mix:

10   50 mM ammonium acetate pH 8.3, 2 mM MgCl₂, 200 mM pyruvate (sodium or ammonium salt), 5 mM alpha-ketoglutarate (sodium salt), 0.05 mM pyridoxal phosphate, deaerated water to achieve a final volume of 1 mL after the addition of the enzymes, 3 mM potassium phosphate, 50 μg/mL of recombinant ProA aldolase (cell extract; total protein concentration of 167 μg/mL), 1000 μg/mL of L-aspartate

15   aminotransferase encoded by the *E. coli aspC* gene (cell extract; total protein concentration of 2500 μg/mL), and solid tryptophan to afford a concentration of > 60 mM (saturated; some undissolved throughout the reaction). The mixture was incubated at 30°C for 4 hours with gentle stirring or mixing.

20   *Substitutions*

The concentration of alpha-ketoglutarate can be reduced to 1 mM and supplemented with 9 mM aspartate with an equivalent yield of monatin. Alternative amino acid acceptors can be utilized in the first step, such as oxaloacetate.

25   When recombinant *L. major* broad substrate aminotransferase was used in place of the *E. coli* L-aspartate aminotransferase, similar yields of monatin were achieved. However, a second unidentified product (3-10% of the major product) with a molecular mass of 292 was also detected by LC-MS analysis. Monatin concentrations of 0.1-0.5 mg/mL were produced when the *E. coli tyrB* encoded enzyme, the *S.*

30   *meliloti tat A* encoded enzyme or the glutamic-oxaloacetic transaminase from pig heart (type IIa) was added as the aminotransferase. When starting the reaction from indole-3-pyruvate, a reductive amination can be done for the last step with glutamate dehydrogenase and NADH (as in Example 7).

The KHG aldolases from *B. subtilis, E. coli,* and *S. meliloti* were also used with the *E. coli* L-aspartate aminotransferase to produce monatin enzymatically. The following reaction conditions were used: 50 mM NH₄-OAc pH 8.3, 2 mM MgCl₂ 200 mM

5   pyruvate, 5 mM glutamate, 0.05 mM pyridoxal phosphate, deaerated water to achieve a final volume of 0.5 mL after the addition of the enzymes, 3 mM potassium phosphate, 20 µg/mL of recombinant *B. subtilis* KHG aldolase (purified), ca. 400 µg/mL of *E. coli* L-aspartate aminotransferase (AspC) unpurified from cell extract, and 12 mM indole-3-pyruvate. The reactions were incubated at 30°C for 30 minutes

10   with shaking. The amount of monatin produced using the *B. subtilis* enzyme was 80 ng/mL, and increased with increasing amounts of aldolase. If indole-3-pyruvate and glutamate were replaced by saturating amounts of tryptophan and 5 mM alpha-ketoglutarate, the production of monatin was increased to 360 ng/mL. Reactions were repeated with 30 µg/mL of each of the three KHG enzymes in 50 mM Tris pH 8.3,

15   with saturating amounts of tryptophan, and were allowed to proceed for an hour in order to increase detection. The *Bacillus* enzyme had the highest activity as in Example 4, producing approximately 4000 ng/mL monatin. The *E. coli* KHG produced 3000 ng/mL monatin, and the *S. meliloti* enzyme produced 2300 ng/mL.

20   **EXAMPLE 7**

Interconversion between MP and Monatin

The amination of MP to form monatin can be catalyzed by aminotransferases such as those identified in Examples 1 and 6, or by dehydrogenases that require a reducing cofactor such as NADH or NADPH. These reactions are reversible and can be

25   measured in either direction. The directionality, when using a dehydrogenase enzyme, can be largely controlled by the concentration of ammonium salts.

Dehydrogenase activity. The oxidative deamination of monatin was monitored by following the increase in absorbance at 340 nm as NAD(P)+ was converted to the

30   more chromophoric NAD(P)H. Monatin was enzymatically produced and purified as described in Example 6.

A typical assay mixture contained 50 mM Tris-HCl, pH 8.0 to 8.9, 0.33 mM NAD$^+$ or

NADP$^+$, 2 to 22 units of glutamate dehydrogenase (Sigma), and 10-15 mM substrate

in 0.2 mL. The assay was performed in duplicate in a UV-transparent microtiter

plate, on a Molecular Devices SpectraMax Plus platereader. A mix of the enzyme,

5   buffer, and NAD(P)$^+$ were pipetted into wells containing the substrate and the

increase in absorbance at 340 nm was monitored at 10 second intervals after brief

mixing. The reaction was incubated at 25°C for 10 minutes. Negative controls were

carried out without the addition of substrate, and glutamate was utilized as a positive

control. The type III glutamate dehydrogenase from bovine liver (Sigma # G-7882)

10   catalyzed the conversion of the monatin to the monatin precursor at a rate of

conversion approximately one-hundredth the rate of the conversion of glutamate to

alpha-ketoglutarate.

Transamination activity. Monatin aminotransferase assays were conducted with the

15   aspartate aminotransferase (AspC) from *E. coli*, the tyrosine aminotransferase (TyrB)

from *E. coli*, the broad substrate aminotransferase (BSAT) from *L. major*, and the two

commercially available porcine glutamate-oxaloacetate aminotransferases described

in Example 1. Both oxaloacetate and alpha-ketoglutarate were tested as the amino

acceptor. The assay mixture contained (in 0.5 mL) 50 mM Tris-HCl, pH 8.0, 0.05

20   mM PLP, 5 mM amino acceptor, 5 mM monatin, and 25 µg of aminotransferase. The

assays were incubated at 30°C for 30 minutes, and the reactions were stopped by

addition of 0.5 mL isopropyl alcohol. The loss of monatin was monitored by LC/MS

(Example 10). The highest amount of activity was noted with *L. major* BSAT with

oxaloacetate as the amino acceptor, followed by the same enzyme with alpha-

25   ketoglutarate as the amino acceptor. The relative activity with oxaloacetate was:

BSAT > AspC > porcine type IIa > porcine type I =TyrB. The relative activity with

alpha-ketoglutarate was: BSAT > AspC > porcine type I > porcine type IIa > TyrB.

EXAMPLE 8

30   *Production of Monatin from Tryptophan and C3 Sources Other than Pyruvate*

As described above in Example 6, indole-3-pyruvate or tryptophan can be converted

to monatin using pyruvate as the C3 molecule. However, in some circumstances,

pyruvate may not be a desirable raw material. For example, pyruvate may be more

expensive than other C3 carbon sources, or may have adverse effects on fermentations

if added to the medium. Alanine can be transaminated by many PLP-enzymes to produce pyruvate.

Tryptophanase-like enzymes perform beta-elimination reactions at faster rates than other PLP enzymes such as aminotransferases. Enzymes from this class (4.1.99.-) can

5   produce ammonia and pyruvate from amino acids such as L-serine, L-cysteine, and derivatives of serine and cysteine with good leaving groups such as O-methyl-L-serine, O-benzyl-L-serine, S-methylcysteine, S-benzylcysteine, S-alkyl-L-cysteine, O-acyl-L-serine, 3-chloro-L-alanine.

10   Processes to produce monatin using EC 4.1.99.- polypeptides can be improved by mutating the β-tyrosinase (TPL) or tryptophanase according to the method of Mouratou et al. (J. Biol. Chem 274:1320-5, 1999). Mouratou et al. describe the ability to covert the β-tyrosinase into a dicarboxylic amino acid β-lyase, which has not been reported to occur in nature. The change in specificity was accomplished by

15   converting valine (V) 283 to arginine (R) and arginine (R) 100 to threonine (T). These amino acid changes allow for the lyase to accept a dicarboxylic amino acid for the hydrolytic deamination reaction (such as aspartate). Aspartate, therefore, can also be used as a source of pyruvate for subsequent aldol condensation reactions.

20   Additionally, cells or enzymatic reactors can be supplied with lactate and an enzyme that converts lactate to pyruvate. Examples of enzymes capable of catalyzing this reaction include lactate dehydrogenase and lactate oxidase.

The reaction mixture consisted of 50 mM Tris-Cl pH 8.3, 2 mM MgCl$_2$, 200 mM C3

25   carbon source, 5 mM alpha-ketoglutarate, sodium salt, 0.05 mM pyridoxal phosphate, deaerated water to achieve a final volume of 0.5 mL after the addition of the enzymes, 3 mM potassium phosphate pH 7.5, 25 µg of crude recombinant C. testosteroni ProA aldolase as prepared as in Example 4, 500 µg of crude L-aspartate aminotransferase (AspC) as prepared in Example 1, and solid tryptophan to afford a concentration of >

30   60 mM (saturated; some undissolved throughout the reaction). The reaction mix was incubated at 30°C for 30 minutes with mixing. Serine, alanine, and aspartate were supplied as 3-carbon sources. Assays were performed with and without secondary PLP enzymes (purified) capable of performing beta-elimination and beta-lyase

reactions (tryptophanase (TNA), double mutant tryptophanase, β-tyrosinase (TPL)).
The results are shown in Table 4:

TABLE 4

Production of monatin utilizing alternative C3-carbon sources

C3-carbon source	Additional PLP Enzyme	Relative Activity
none	None	0%
pyruvate	None	100%
serine	None	3%
serine	11 μg wildtype TNA (1 U)	5.1%
serine	80 μg double mutant TNA	4.6%
alanine	None	32%
alanine	11 μg wildtype TNA	41.7%
alanine	80 μg mutant TNA	43.9%
aspartate	110 μg wildtype TNA (10 U)	7.7%
aspartate	5 U wildtype TPL (crude)	5.1%
aspartate	80 μg mutant TNA	3.3%

5   The monatin produced from alanine and serine as 3-carbon sources was verified by
LC/MS/MS daughter scan analysis, and was identical to the characterized monatin
produced in Example 6. Alanine was the best alternative tested, and was
transaminated by the AspC enzyme. The amount of monatin produced was increased
10   by addition of the tryptophanase, which is capable of transamination as a secondary
activity. The amount of monatin produced with serine as a carbon source nearly
doubled with the addition of the tryptophanase enzymes, even though only one-fifth
of the amount of tryptophanase was added in comparison to the aminotransferase.
AspC is capable of some amount of beta-elimination activity alone. The results with
15   aspartate indicate that the tryptophanase activity on aspartate does not increase with
the same site-directed mutations as previously suggested for β-tyrosinase. It is
expected that the mutant β-tyrosinase will have higher activity for production of
monatin.

20   **EXAMPLE 9**

Chemical Synthesis of Monatin
The addition of alanine to indole-3-pyruvic acid produces monatin, and this reaction
can be performed synthetically with a Grignard or organolithium reagent.

For example, to 3-chloro- or 3-bromo-alanine which has been appropriately blocked at the carboxyl and amino groups, is added magnesium under anhydrous conditions. Indole-3-pyruvate (appropriately blocked) is then added to form the coupled product followed by removal of the protecting groups to form monatin. Protecting groups that

5   are particularly useful include THP (tetrahydropyranyl ether) which is easily attached and removed.

EXAMPLE 10

Detection of Tryptophan, Monatin, and MP

10   This example describes methods used to detect the presence of monatin, or its precursor 2-hydroxy 2-(indol-3-ylmethyl)-4-keto glutaric acid.

LC/MS Analysis

Analyses of mixtures for monatin, MP, and/or tryptophan derived from *in vitro* or *in*

15   *vivo* biochemical reactions were performed using a Waters/Micromass liquid chromatography-tandem mass spectrometry (LC/MS/MS) instrument including a Waters 2690 liquid chromatograph with a Waters 996 Photo-Diode Array (PDA) absorbance monitor placed in series between the chromatograph and a Micromass Quattro Ultima triple quadrupole mass spectrometer. LC separations were made

20   using a Supelco Discovery C_{18} reversed-phase chromatography column, 2.1mm x 150 mm, or an Xterra MS C_8 reversed-phase chromatography column, 2.1mm x 250 mm, at room temperature. The LC mobile phase consisted of A) water containing 0.05% (v/v) trifluoroacetic acid and B) methanol containing 0.05% (v/v) trifluoroacetic acid.

25   The gradient elution was linear from 5% B to 35% B, 0-9 min, linear from 35% B to 90% B, 9-16 min, isocratic at 90% B, 16-20 min, linear from 90% B to 5% B, 20-22 min, with a 10 min re-equilibration period between runs. The flow rate was 0.25 mL/min, and PDA absorbance was monitored from 200 nm to 400 nm. All parameters of the ESI-MS were optimized and selected based on generation of

30   protonated molecular ions ($[M + H]^+$) of the analytes of interest, and production of characteristic fragment ions.

The following instrumental parameters were used for LC/MS analysis of monatin: Capillary: 3.5 kV; Cone: 40 V; Hex 1: 20 V; Aperture: 0 V; Hex 2: 0 V; Source

temperature: 100°C; Desolvation temperature: 350°C; Desolvation gas: 500 L/h; Cone gas: 50 L/h; Low mass resolution (Q1): 15.0; High mass resolution (Q1): 15.0; Ion energy: 0.2; Entrance: 50V; Collision Energy: 2; Exit: 50V; Low mass resolution (Q2): 15; High mass resolution (Q2): 15; Ion energy (Q2): 3.5; Multiplier: 650.

5   Uncertainties for reported mass/charge ratios (*m/z*) and molecular masses are ± 0.01%. Initial detection of the alpha-keto acid form of monatin (MP) and monatin in the mixtures was accomplished by LC/MS monitoring with collection of mass spectra for the region *m/z* 150–400. Selected ion chromatograms for protonated molecular ions ($[M + H]^+ = 292$ for MP, $[M + H]^+ = 293$ for monatin) allowed direct
10   identification of these analytes in the mixtures.

MS/MS Analysis

LC/MS/MS daughter ion experiments were performed on monatin as follows. A daughter ion analysis involves transmission of the parent ion (e.g., $m/z = 293$ for
15   monatin) of interest from the first mass analyzer (Q1) into the collision cell of the mass spectrometer, where argon is introduced and chemically dissociates the parent into fragment (daughter) ions. These fragment ions are then detected with the second mass analyzer (Q2), and can be used to corroborate the structural assignment of the parent. Tryptophan was characterized and quantified in the same way via
20   transfmission and fragmentation of $m/z = 205$.

The following instrumental parameters were used for LC/MS/MS analysis of monatin: Capillary: 3.5 kV; Cone: 40 V; Hex 1: 20 V; Aperture: 0 V; Hex 2: 0 V; Source temperature: 100 °C; Desolvation temperature: 350 °C; Desolvation gas: 500 L/h;
25   Cone gas: 50 L/h; Low mass resolution (Q1): 13.0; High mass resolution (Q1): 13.0; Ion energy: 0.2; Entrance: -5 V; Collision Energy: 14; Exit: 1V; Low mass resolution (Q2): 15; High mass resolution (Q2): 15; Ion energy (Q2): 3.5; Multiplier: 650.

High-Throughput Determination of Monatin and Tryptophan

30   High-throughput analyses (< 5 min/sample) of mixtures for monatin and tryptophan derived from *in vitro* or *in vivo* reactions were carried out using instrumentation described above, and the same parameters as described for LC/MS/MS. LC separations were made using a 4.6 mm x 50 mm Advanced Separation Technologies

Chirobiotic T column at room temperature. The LC mobile phase consisted of A)

water containing 0.25% acetic acid; B) Methanol containing 0.25% acetic acid. The

isocratic elution was at 50% B, 0-5 min. The flow rate was 0.6 mL/min. All

parameters of the ESI-MS/MS system were optimized and selected based on optimal

5   in-source generation of the protonated molecular ion of tryptophan and the internal

standard $^2H_5$-tryptophan, as well as collision-induced production of amino acid-

specific fragment ions for multiple reaction monitoring (MRM) experiments (Table

1). The following instrumental parameters were used for LC/MS/MS analysis of

monatin and tryptophan in the positive ion multiple reaction monitoring (mrm) mode:

10   Capillary: 3.5 kV; Cone: 20 V; Hex 1: 15 V; Aperture: 1 V; Hex 2: 0 V; Source

temperature: 100 °C; Desolvation temperature: 350 °C; Desolvation gas: 500 L/h;

Cone gas: 40 L/h; Low mass resolution (Q1): 12.0; High mass resolution (Q1): 12.0;

Ion energy: 0.2; Entrance: - 5 V; Collision Energy: 14; Exit: 1 V; Low mass

resolution (Q2): 15; High mass resolution (Q2): 15; Ion energy (Q2): 0.5; Multiplier:

15   650. MRM parameters: Interchannel delay: 0.03 s; Interscan delay: 0.03 s; Dwell:

0.05 s.

Accurate Mass Measurement of Monatin.

High resolution MS analysis was carried out using an Applied Biosystems-Perkin

20   Elmer Q-Star hybrid quadrupole/time-of-flight mass spectrometer. The measured

mass for protonated monatin used tryptophan as an internal mass calibration standard.

The calculated mass of protonated monatin, based on the elemental composition

$C_{14}H_{17}N_2O_5$ is 293.1137. Monatin produced using the biocatalytic process described

in Example A showed a measured mass of 293.1144. This is a mass measurement

25   error of less than 2 parts per million (ppm), providing conclusive evidence of the

elemental composition of monatin produced enzymatically.

EXAMPLE 11

Production of Monatin in Bacteria

30   This example describes methods used to produce monatin in *E. coli* cells. One skilled

in the art will understand that similar methods can be used to produce monatin in

other bacterial cells. In addition, vectors containing other genes in the monatin

synthesis pathway (FIG. 2) can be used.

Trp-1 + glucose medium, a minimal medium that has been used for increased

production of tryptophan in *E. coli* cells (Zeman *et al. Folia Microbiol.* 35:200-4,

1990), was prepared as follows. To 700 mL nanopure water the following reagents

5   were added: 2 g $(NH_4)_2SO_4$, 13.6 g KH_2PO_4, 0.2 g $MgSO_4*7H_2O$, 0.01 g $CaCl_2*2H_2O$,

and 0.5 mg $FeSO_4*7H_2O$. The pH was adjusted to 7.0, the volume was increased to

850 mL, and the medium was autoclaved. A 50% glucose solution was prepared

separately, and sterile-filtered. Forty mL was added to the base medium (850 mL) for

a 1 L final volume.

10

A 10 g/L L-tryptophan solution was prepared in 0.1 M sodium phosphate pH 7, and

sterile-filtered. One-tenth volume was typically added to cultures as specified below.

A 10% sodium pyruvate solution was also prepared and sterile-filtered. A 10 mL

aliquot was typically used per liter of culture. Stocks of ampicillin (100 mg/mL),

15   kanamycin (25 mg/mL) and IPTG (840 mM) were prepared, sterile-filtered, and

stored at –20°C before use. Tween 20 (polyoxyethylene 20-Sorbitan monolaurate)

was utilized at a 0.2% (vol/vol) final concentration. Ampicillin was used at non-lethal

concentrations, typically 1-10 µg/mL final concentration.

20   Fresh plates of *E. coli* BL21(DE3)::*C. testosteroni proA*/pET 30 Xa/LIC (described in

Example 4) were prepared on LB medium containing 50 µg/mL kanamycin.

Overnight cultures (5 mL) were inoculated from a single colony and grown at 30°C in

LB medium with kanamycin. Typically a 1 to 50 inoculum was used for induction in

trp-1 + glucose medium. Fresh antibiotic was added to a final concentration of 50

25   mg/L. Shake flasks were grown at 37°C prior to induction.

Cells were sampled every hour until an OD_{600} of 0.35-0.8 was obtained. Cells were

then induced with 0.1 mM IPTG, and the temperature reduced to 34 °C. Samples (1

ml) were collected prior to induction (zero time point) and centrifuged at 5000 x g.

30   The supernatant was frozen at –20°C for LC/MS analysis. Four hours post-induction,

another 1 mL sample was collected, and centrifuged to separate the broth from the

cell pellet. Tryptophan, sodium pyruvate, ampicillin, and Tween were added as

described above.

The cells were grown for 48 hours post-induction, and another 1 mL sample was taken and prepared as above. At 48 hours, another aliquot of tryptophan and pyruvate were added. The entire culture volume was centrifuged after approximately 70 hours

5   of growth (post-induction), for 20 minutes at 4°C and 3500 rpm. The supernatant was decanted and both the broth and the cells were frozen at –80°C. The broth fractions were filtered and analyzed by LC/MS. The heights and areas of the $[M+H]^+ = 293$ peaks were monitored as described in Example 10. The background level of the medium was subtracted. The data was also normalized for cell growth by plotting the

10   height of the $[M+H]^+ = 293$ peak divided by the optical density of the culture at 600 nm.

Higher levels of monatin were produced when pyruvate, ampicillin, and Tween were added 4 hours post induction rather than at induction. Other additives such as PLP,

15   additional phosphate, or additional $MgCl_2$ did not increase the production of monatin. Higher titers of monatin were obtained when tryptophan was utilized instead of indole-3-pyruvate, and when the tryptophan was added post-induction rather than at inoculation, or at induction. Prior to induction, and 4 hours post-induction (at time of substrate addition), there was typically no detectable level of monatin in the

20   fermentation broth or cellular extracts. Negative controls were done utilizing cells with pET30a vector only, as well as cultures where tryptophan and pyruvate were not added. A parent MS scan demonstrated that the compound with $(m+1)/z = 293$ was not derived from larger molecules, and daughter scans (performed as in Example 10) were similar to monatin made *in vitro*.

25

The effect of Tween was studied by utilizing 0, 0.2% (vol/vol), and 0.6% final concentrations of Tween-20. The highest amount of monatin produced by shake flasks was at 0.2% Tween. The ampicillin concentration was varied between 0 and 10 μg/mL. The amount of monatin in the cellular broth increased rapidly (2.5 X)

30   between 0 and 1 μg/mL, and increased 1.3 X when the ampicillin concentration was increased from 1 to 10 μg/mL.

A time course experiment showing typical results is shown in FIG. 10. The amount
of monatin secreted into the cell broth increased, even when the values are normalized
for cell growth. By using the molar extinction coefficient of tryptophan, the amount
of monatin in the broth was estimated to be less than 10 µg/mL. The same

5   experiment was repeated with the cells containing vector without *proA* insert. Many
of the numbers were negative, indicating the peak height at m/z=293 was less in these
cultures than in the medium alone (FIG. 10). The numbers were consistently lower
when tryptophan and pyruvate were absent, demonstrating that monatin production is
a result of an enzymatic reaction catalyzed by the aldolase enzyme.

10

The *in vivo* production of monatin in bacterial cells was repeated in 800 mL shake
flask experiments and in fermentors. A 250 mL sample of monatin (in cell-free broth)
was purified by anion exchange chromatography and preparative reverse-phase liquid
chromatography. This sample was evaporated, and submitted for high resolution

15   mass analysis (described in Example 6). The high resolution MS indicated that the
metabolite being produced is monatin.

In vitro assays indicate that aminotransferase needs to be present at higher levels than
aldolase (see Example 6), therefore the aspartate aminotransferase from *E. coli* was

20   overexpressed in combination with the aldolase gene to increase the amount of
monatin produced. Primers were designed to introduce *C. testosteroni proA* into an
operon with *aspC*/pET30 Xa/LIC, as follows: 5' primer:
ACTCGGATCCGAAGGAGATATACATATGTACGAACTGGGACT (SEQ ID
NO: 67) and 3' primer: CGGCTGTCGACCGTTAGTCAATATATTTCAGGC (SEQ

25   ID NO: 68). The 5' primer contains a BamHI site, the 3' primer contains a SalI site
for cloning. PCR was performed as described in Example 4, and gel purified. The
aspC/pET30 Xa/LIC construct was digested with BamHI and SalI, as was the PCR
product. The digests were purified using a Qiagen spin column. The *proA* PCR
product was ligated to the vector using the Roche Rapid DNA Ligation kit

30   (Indianapolis, IN) according to manufacturer's instructions. Chemical
transformations were done using Novablues Singles (Novagen) as described in
Example 1. Colonies were grown up in LB medium containing 50 mg/L kanamycin
and plasmid DNA was purified using the Qiagen spin miniprep kit. Clones were

screened by restriction digest analysis and sequence was confirmed by Seqwright (Houston, TX). Constructs were subcloned into BLR(DE3), BLR(DE3)pLysS, BL21(DE3) and BL21(DE3)pLysS (Novagen). The *proA*/pET30 Xa/LIC construct was also transformed into BL21(DE3)pLysS.

Initial comparisons of BLR(DE3) shake flask samples under the standard conditions described above demonstrated that the addition of the second gene (*aspC*) improved the amount of monatin produced by seven-fold. To hasten growth, BL21(DE3)-derived host strains were used. The *proA* clones and the two gene operon clones were induced in Trp-1 medium as above, the pLysS hosts had chloramphenicol (34 mg/L) added to the medium as well. Shake flask experiments were performed with and without the addition of 0.2% Tween-20 and 1 mg/L ampicillin. The amount of monatin in the broth was calculated using *in vitro* produced purified monatin as a standard. SRM analyses were performed as described in Example 10. Cells were sampled at zero, 4 hours, 24 hours, 48 hours, 72 hours, and 96 hours of growth.

The results are shown in Table 5 for the maximum amounts produced in the culture broths. In most instances, the two gene construct gave higher values than the *proA* construct alone. The pLysS strains, which should have leakier cell envelopes, had higher levels of monatin secreted, even though these strains typically grow at a slower rate. The additions of Tween and ampicillin were beneficial.

Table 5

Amount of Monatin Produced by *E. coli* Bacteria

Construct	Host	Tween + Amp	µg/mL monatin	time
proA	BL21(DE3)	-	0.41	72 hr
proA	BL21(DE3)	+	1.58	48 hr
proA	BL21(DE3)pLysS	-	1.04	48 hr
proA	BL21(DE3)pLysS	+	1.60	48 hr
aspC:proA	BL21(DE3)	-	0.09	48 hr
aspC:proA	BL21(DE3)	+	0.58	48 hr
aspC:proA	BL21(DE3)pLysS	-	1.39	48 hr
aspC:proA	BL21(DE3)pLysS	+	6.68	48 hr

EXAMPLE 12

Production of Monatin in Yeast

This example describes methods used to produce monatin in eukaryotic cells. One skilled in the art will understand that similar methods can be used to produce monatin in any cell of interest. In addition, other genes can be used (e.g., those listed in FIG. 2) in addition to, or alternatively to those described in this example.

5

The pESC Yeast Epitope Tagging Vector System (Stratagene, La Jolla, CA) was used to clone and express the *E. coli aspC* and *C. testosteroni proA* genes into *Saccharomyces cerevisiae*. The pESC vectors contain both the GAL1 and the GAL10 promoters on opposite strands, with two distinct multiple cloning sites, allowing for

10 expression of two genes at the same time. The pESC-His vector also contains the *His3* gene for complementation of histidine auxotrophy in the host (YPH500). The GAL1 and GAL10 promoters are repressed by glucose and induced by galactose; a Kozak sequence is utilized for optimal expression in yeast. The pESC plasmids are shuttle vectors, allowing the initial construct to be made in *E. coli* (with the *bla* gene

15 for selection); however, no bacterial ribosome binding sites are present in the multiple cloning sites.

The following primers were designed for cloning into pESC-His (restriction sites are underlined, Kozak sequence is in bold): *aspC* (*Bam*HI/*Sal*I), GAL1: 5'-

20 CGC<u>GGATCC</u>**ATAATGG**TTGAGAACATTACCG-3' (SEQ ID NO: 69) and 5'-ACGC<u>GTCGAC</u>TTACAGCACTGCCACAATCG-3' (SEQ ID NO: 70). *proA* (*Eco*RI/*Not*I), GAL10: 5'-CCG<u>GAATTC</u>**ATAATGG**TCGAACTGGGAGTTGT-3' (SEQ ID NO: 71) and 5'-GAAT<u>GCGGCCGC</u>TTAGTCAATATATTTCAGGCC-3' (SEQ ID NO: 72).

25

The second codon for both mature proteins was changed from an aromatic amino acid to valine due to the introduction of the Kozak sequence. The genes of interest were amplified using pET30 Xa/LIC miniprep DNA from the clones described in Examples 1 and Example 4 as template. PCR was performed using an Eppendorf Master cycler

30 gradient thermocycler and the following protocol for a 50 μL reaction: 1.0 μL template, 1.0 μM of each primer, 0.4 mM each dNTP, 3.5 U Expand High Fidelity Polymerase (Roche, Indianapolis, IN), and 1X Expand™ buffer with Mg. The thermocycler program used consisted of a hot start at 94°C for 5 minutes, followed by

29 repetitions of the following steps: 94°C for 30 seconds, 50°C for 1 minute 45 seconds, and 72°C for 2 minutes 15 seconds. After the 29 repetitions the sample was maintained at 72°C for 10 minutes and then stored at 4°C. The PCR products were purified by separation on a 1% TAE-agarose gel followed by recovery using a

5   QIAquick Gel Extraction Kit (Qiagen, Valencia, CA).

The pESC-His vector DNA (2.7 µg) was digested with *BamHI/SalI* and gel-purified as above. The *aspC* PCR product was digested with *BamHI/SalI* and purified with a QIAquick PCR Purification Column. Ligations were performed with the Roche

10   Rapid DNA Ligation Kit following the manufacturer's protocols. Desalted ligations were electroporated into 40 µl Electromax DH10B competent cells (Invitrogen) in a 0.2 cm Biorad disposable cuvette using a Biorad Gene Pulser II with pulse controller plus, according to the manufacturer's instructions. After 1 hour of recovery in 1 mL of SOC medium, the transformants were plated on LB medium containing 100 µg/mL

15   ampicillin. Plasmid DNA preparations for clones were done using QIAprep Spin Miniprep Kits. Plasmid DNA was screened by restriction digest, and sequenced (Seqwright) for verification using primers designed for the vector.

The *aspC* /pESC-His clone was digested with *Eco*RI and *Not*I, as was the *proA* PCR

20   product. DNA was purified as above, and ligated as above. The two gene construct was transformed into DH10B cells and screened by restriction digest and DNA sequencing.

The construct was transformed into *S. cerevisiae* strain YPH500 using the S.c.

25   EasyComp™ Transformation Kit (Invitrogen). Transformation reactions were plated on SC-His minimal medium (Invitrogen pYES2 manual) containing 2% glucose. Individual yeast colonies were screened for the presence of the *proA* and *aspC* genes by colony PCR using the PCR primers above. Pelleted cells (2 µl) were suspended in 20 µL of Y-Lysis Buffer (Zymo Research) containing 1 µl of zymolase and heated at

30   37°C for 10 minutes. Four µL of this suspension was then used in a 50 µL PCR reaction using the PCR reaction mixture and program described above.

Five mL cultures were grown overnight on SC-His + glucose at 30°C and 225 rpm. The cells were gradually adjusted to growth on raffinose in order to minimize the lag period prior to induction with galactose. After approximately 12 hours of growth, absorbance measurements at 600 nm were taken, and an appropriate volume of cells

5   was spun down and resuspended to give an OD of 0.4 in the fresh SC-His medium. The following carbon sources were used sequentially: 1% raffinose + 1 % glucose, 0.5% glucose + 1.5% raffinose, 2% raffinose, and finally 1% raffinose + 2% galactose for induction.

10   After approximately 16 hours of growth in induction medium, the 50 mL cultures were divided into duplicate 25 mL cultures, and the following were added to only one of the duplicates: (final concentrations) 1 g/L L-tryptophan, 5 mM sodium phosphate pH 7.1, 1 g/L sodium pyruvate, 1 mM MgCl₂. Samples of broths and cell pellets from the non-induction medium, and from the 16 hour cultures prior to addition of

15   substrates for the monatin pathway, were saved as negative controls. In addition, constructs containing only a functional *aspC* gene (and a truncated *proA* gene) were utilized as another negative control. The cells were allowed to grow for a total of 69 hours post-induction. Occasionally the yeast cells were induced at a lower OD, and only grown for 4 hours prior to addition of tryptophan and pyruvate. However, these

20   monatin substrates appear to inhibit growth and the addition at higher OD was more effective.

The cell pellets from the cultures were lysed with 5 mL of YeastBuster™ + 50 μl THP (Novagen) per gram (wet weight) of cells following manufacturer's protocols,

25   with the addition of protease inhibitors and benzonase nuclease as described in previous examples. The culture broth and cell extracts were filtered and analyzed by SRM as described in Example 10. Using this method, no monatin was detected in the broth samples, indicating that the cells could not secrete monatin under these conditions. The proton motive force may be insufficient under these conditions or the

30   general amino acid transporters may be saturated with tryptophan. Protein expression was not at a level that allowed for detection of changes using SDS-PAGE.

Monatin was detectable (approximately 60 ng/mL) transiently in cell extracts of the culture with two functional genes, when tryptophan and pyruvate were added to the medium. Monatin was not detected in any of the negative control cell extracts. *In vitro* assays for monatin were performed in duplicate with 4.4 mg/mL of total protein
5   (about double what is typically used for *E. coli* cell extracts) using the optimized assay described in Example 6. Other assays were performed with the addition of either 32 µg/mL *C. testosteroni* ProA aldolase or 400 µg/mL AspC aminotransferase, to determine which enzyme was limiting in the cell extract. Negative controls were performed with no addition of enzyme, or the addition of only AspC aminotransferase
10   (the aldol condensation can occur to some extent without enzyme). Positive controls were performed with partially pure enzymes (30-40%), using 16 µg/mL aldolase and 400 µg/mL aminotransferase.

In vitro results were analyzed by SRM. The analysis of cell extracts showed that
15   tryptophan was effectively transported into the cells when it was added to the medium post-induction, resulting in tryptophan levels two orders of magnitude higher than those in which no additional tryptophan was added. The results for *in vitro* monatin analysis are shown in Table 6 (numbers indicate ng/mL).

20   **Table 6**

Monatin production with yeast cell extracts

	aspC construct	+ aldolase	+ AspC	two-gene construct	+ aldolase	+ AspC
repressed (glucose medium)	0	888.3	173.5	0	465.2	829
24 hr induced	0	2832.8	642.4	0	1375.6	9146.6
69 hr induced	0	4937.3	340.3	71.9	1652.8	23693.5
69 hr + subs.	0	556.9	659.1	21.9	755.6	16688.2
+ control (purified enzymes)	21853			21853		
-control (no enzymes)	0		254.3	0		254.3

Positive results were obtained with the full two-gene construct cell extracts with and without substrate added to the growth medium. These results, in comparison to the
25   positive controls, indicate that the enzymes were expressed at levels of close to 1% of the total protein in yeast. The amount of monatin produced when the cell extract of

the *aspC* construct (with truncated *proA*) was assayed with aldolase was significantly
greater than when cell extracts were assayed alone, and indicates that the recombinant
AspC aminotransferase comprises approximately 1-2% of the yeast total protein. The
cell extracts of uninduced cultures had a small amount of activity when assayed with

5   aldolase due to the presence of native aminotransferases in the cells. When assayed
with AspC aminotransferase, the activity of the extracts from uninduced cells
increased to the amount of monatin produced by the negative control with AspC (ca.
200 ng/ml). In contrast, the activity observed when assaying the two gene construct
cell extract increases more when aminotransferase is supplemented than when

10   aldolase is added. Since both genes should be expressed at the same level, this
indicates that the amount of monatin produced is maximized when the level of
aminotransferase is higher than that of aldolase, in agreement with results shown in
Example 6.

15   The addition of pyruvate and tryptophan not only inhibits cellular growth, but
apparently inhibits protein expression as well. The addition of the pESC-Trp plasmid
can be used to correct for tryptophan auxotrophy of the YPH500 host cells, to provide
a means of supplying tryptophan with fewer effects on growth, expression, and
secretion.

20

EXAMPLE 13

Improvement of Enzymatic Processes using Coupled Reactions

In theory, if no side reactions or degradation of substrates or intermediates occurs, the
maximum amount of product formed from the enzymatic reaction illustrated in FIG. 1

25   is directly proportional to the equilibrium constants of each reaction, and the
concentrations of tryptophan and pyruvate. Tryptophan is not a highly soluble
substrate, and concentrations of pyruvate greater than 200 mM appear to have a
negative effect on the yield (see Example 6).

30   Ideally, the concentration of monatin is maximized with respect to substrates, in order
to decrease the cost of separation. Physical separations can be performed such that
the monatin is removed from the reaction mixture, preventing the reverse reactions
from occuring. The raw materials and catalysts can then be regenerated. Due to the
similarity of monatin in size, charge, and hydrophobicity to several of the reagents

and intermediates, physical separations will be difficult unless there is a high amount of affinity for monatin (such as an affinity chromatography technique). However, the monatin reactions can be coupled to other reactions such that the equilibrium of the system is shifted toward monatin production. The following are examples of

5   processes for improving the yield of monatin obtained from tryptophan or indole-3-pyruvate.

Coupled reactions using oxaloacetate decarboxylase (EC 4.1.1.3)

FIG. 11 is an illustration of the reaction. Tryptophan oxidase and catalase are utilized

10 to drive the reaction in the direction of indole-3-pyruvate production. Catalase is used in excess such that hydrogen peroxide is not available to react in the reverse direction or to damage the enzymes or intermediates. Oxygen is regenerated during the catalase reaction. Alternatively, indole-3-pyruvate can be used as the substrate.

15 Aspartate is used as the amino donor for the amination of MP, and an aspartate aminotransferase is utilized. Ideally, an aminotransferase that has a low specificity for the tryptophan/indole-3-pyruvate reaction in comparison to the MP to monatin reaction is used so that the aspartate is not utilized to reaminate the indole-3-pyruvate. Oxaloacetate decarboxylase (from *Pseudomonas sp.*) can be added to convert the

20 oxaloacetate to pyruvate and carbon dioxide. Since CO_2 is volatile, it is not available for reaction with the enzymes, decreasing or even preventing the reverse reactions. The pyruvate produced in this step can also be utilized in the aldol condensation reaction. Other decarboxylase enzymes can be used, and homologs are known to exist in *Actinobacillus actinomycetemcomitans, Aquifex aeolicus, Archaeoglobus*

25 *fulgidus, Azotobacter vinelandii, Bacteroides fragilis*, several *Bordetella* species, *Campylobacter jejuni, Chlorobium tepidum, Chloroflexus aurantiacus, Enterococcus faecalis, Fusobacterium nucleatum, Klebsiella pneumoniae, Legionella pneumophila, Magnetococcus MC-1, Mannheimia haemolytica, Methylobacillus flagellatus KT, Pasteurella multocida* Pm70, *Petrotoga miotherma, Porphyromonas gingivalis*,

30 several *Pseudomonas* species, several *Pyrococcus* species, *Rhodococcus*, several *Salmonella* species, several *Streptococcus* species, *Thermochromatium tepidum, Thermotoga maritima, Treponema pallidum*, and several *Vibrio* species.

Tryptophan aminotransferase assays were performed with the aspartate
aminotransferase (AspC) from *E. coli*, the tyrosine aminotransferase (TyrB) from *E.
coli*, the broad substrate aminotransferase (BSAT) from *L. major*, and the two
commercially available porcine glutamate-oxaloacetate aminotransferases as

5   described in Example 1. Both oxaloacetate and alpha-ketoglutarate were tested as the
amino acceptor. The ratio of activity using monatin (Example 7) versus activity using
tryptophan was compared, to determine which enzyme had the highest specificity for
the monatin aminotransferase reaction. These results indicated that the enzyme with
the highest specificity for the monatin reaction verses the tryptophan reaction is the

10 Porcine type II-A glutamate-oxaloacetate aminotransferase, GOAT (Sigma G7005).
This specificity was independent of which amino acceptor was utilized. Therefore,
this enzyme was used in the coupled reactions with oxaloacetate decarboxylase.

A typical reaction starting from indole-3-pyruvate included (final concentrations) 50

15 mM Tris-Cl pH 7.3, 6 mM indole-3-pyruvate, 6 mM sodium pyruvate, 6 mM
aspartate, 0.05 mM PLP, 3 mM potassium phosphate, 3 mM $MgCl_2$, 25 μg/mL
aminotransferase, 50 μg/mL *C. testosteroni* ProA aldolase, and 3 Units/mL of
decarboxylase (Sigma O4878). The reactions were allowed to proceed for 1 hour at
26°C. In some cases, the decarboxylase was omitted or the aspartate was substituted

20 with alpha-ketoglutarate (as negative controls). The aminotransferase enzymes
described above were also tested in place of the GOAT to confirm earlier specificity
experiments. Samples were filtered and analyzed by LC/MS as described in Example
10. The results demonstrate that the GOAT enzyme produced the highest amount of
monatin per mg of protein, with the least amount of tryptophan produced as a

25 byproduct. In addition, there was a 2-3 fold benefit from having the decarboxylase
enzyme added. The *E. coli* AspC enzyme also produced large amounts of monatin in
comparison to the other aminotransferases.

Monatin production was increased by: 1) periodically adding 2 mM additions of

30 indole-pyruvate, pyruvate, and aspartate (every half hour to hour), 2) performing the
reactions in an anaerobic environment or with degassed buffers, 3) allowing the
reactions to proceed overnight, and 4) using freshly prepared decarboxylase that has
not been freeze-thawed multiple times. The decarboxylase was inhibited by

concentrations of pyruvate greater than 12 mM. At concentrations of indole-3-pyruvate higher than 4 mM, side reactions with indole-3-pyruvate were hastened. The amount of indole-3-pyruvate used in the reaction could be increased if the amount of aldolase was also increased. High levels of phosphate (50 mM) and aspartate (50

5   mM) were found to be inhibitory to the decarboxylase enzyme. The amount of decarboxylase enzyme added could be reduced to 0.5 U/mL with no decrease in monatin production in a one hour reaction. The amount of monatin produced increased when the temperature was increased from 26°C to 30°C and from 30°C to 37°C; however, at 37°C the side reactions of indole-3-pyruvate were also hastened.

10   The amount of monatin produced increased with increasing pH from 7 to 7.3, and was relatively stable from pH 7.3-8.3.

A typical reaction starting with tryptophan included (final concentrations) 50 mM Tris-Cl pH 7.3, 20 mM tryptophan, 6 mM aspartate, 6 mM sodium pyruvate, 0.05 mM

15   PLP, 3 mM potassium phosphate, 3 mM $MgCl_2$, 25 μg/mL aminotransferase, 50 μg/mL *C. testosteroni* ProA aldolase, 4 Units/mL of decarboxylase, 5-200 mU/mL L-amino acid oxidase (Sigma A-2805), 168 U/mL catalase (Sigma C-3515), and 0.008 mg FAD. Reactions were carried out for 30 minutes at 30°C. Improvement was observed with the addition of decarboxylase. The greatest amount of monatin was

20   produced when 50 mU/mL of oxidase was used. Improvements were similar to those observed when indole-3-pyruvate was used as the substrate. In addition, the amount of monatin produced increased when 1) the tryptophan level was low (i.e., below the K_m of the aminotransferase enzyme and therefore unable to compete with MP in the active site), and 2) the ratio of oxidase to aldolase and aminotransferase was

25   maintained at a level such that indole-3-pyruvate could not accumulate.

Whether starting with either indole-3-pyruvate or tryptophan, the amount of monatin produced in assays with incubation times of 1-2 hours increased when 2-4 times the amounts of all the enzymes were used while maintaining the same enzyme ratio.

30   Using either substrate, concentrations of approximately 1 mg/mL of monatin were achieved. The amount of tryptophan produced if starting from indole-pyruvate was typically less than 20% of the amount of product, which shows the benefit of utilizing

coupled reactions. With further optimization and control of the concentrations of intermediates and side reactions, the productivity and yield can be improved greatly.

Coupled reactions using lysine epsilon aminotransferase (EC 2.6.1.36)

5   Lysine epsilon aminotransferase (L-Lysine 6-transaminase) is found in several organisms, including *Rhodococcus, Mycobacterium, Streptomyces, Nocardia, Flavobacterium, Candida utilis*, and *Streptomyces*. It is utilized by organisms as the first step in the production of some beta-lactam antibiotics (Rius and Demain, *J. Microbiol. Biotech.*, 7:95-100, 1997). This enzyme converts lysine to L-2-

10   aminoadipate 6-semialdehyde (allysine), by a PLP-mediated transamination of the C-6 of lysine, utilizing alpha-ketoglutarate as the amino acceptor. Allysine is unstable and spontaneously undergoes an intramolecular dehydration to form 1-piperideine 6-carboxylate, a cyclic molecule. This effectively inhibits any reverse reaction from occuring. The reaction scheme is depicted in FIG. 12. An alternative enzyme, lysine-

15   pyruvate 6-transaminase (EC 2.6.1.71), can also be used.

A typical reaction contained in 1 mL: 50 mM Tris-HCl pH 7.3, 20 mM indole-3-pyruvate, 0.05 mM PLP, 6 mM potassium phosphate pH 8, 2-50 mM sodium pyruvate, 1.5 mM MgCl$_2$, 50 mM lysine, 100 µg aminotransferase (lysine epsilon

20   aminotransferase LAT-101, BioCatalytics Pasadena, CA), and 200 µg *C. testosteroni* ProA aldolase. The amount of monatin produced increased with increasing concentrations of pyruvate. The maximum amount using these reaction conditions (at 50 mM pyruvate) was 10-fold less than what was observed with coupled reactions using oxaloacetate decarboxylase (approximately 0.1 mg/mL).

25

A peak with $[M+H]^+$ = 293 eluted at the expected time for monatin and the mass spectrum contained several of the same fragments observed with other enzymatic processes. A second peak with the correct mass to charge ratio (293) eluted slightly earlier than what is typically observed for the S,S monatin produced in Example 6,

30   and may indicate the presence of another isomer of monatin. Very little tryptophan was produced by this enzyme. However, there is likely some activity on pyruvate (producing alanine as a byproduct). Also, the enzyme is known to be unstable. Improvements can be made by performing directed evolution experiments to increase

stability, reduce the activity with pyruvate, and increase the activity with MP. These reactions can also be coupled to L-amino acid oxidase/catalase as described above.

Other coupled reactions

5   Another coupling reaction that can improve monatin yield from tryptophan or indole-pyruvate is shown in FIG. 13. Formate dehydrogenase (EC 1.2.1.2 or 1.2.1.43) is a common enzyme. Some formate dehydrogenases require NADH while others can utilize NADPH. Glutamate dehydrogenase catalyzed the interconversion between the monatin precursor and monatin in previous examples, using ammonium based buffers.

10   The presence of ammonium formate and formate dehydrogenase is an efficient system for regeneration of cofactors, and the production of carbon dioxide is an efficient way to decrease the rate of the reverse reactions (Bommarius *et al.*, *Biocatalysis* 10:37, 1994 and Galkin *et al. Appl. Environ. Microbiol.* 63:4651-6, 1997). In addition, large amounts of ammonium formate can be dissolved in the reaction buffer. The yield of

15   monatin produced by glutamate dehydrogenase reactions (or similar reductive aminations) can be improved by the addition of formate dehydrogenase and ammonium formate.

Other processes can be used to drive the equilibrium toward monatin production. For

20   instance, if aminopropane is utilized as the amino acid donor in the conversion of MP to monatin with an omega-amino acid aminotransferase (EC 2.6.1.18) such as those described by in US patents 5,360,724 and 5,300,437, one of the resulting products would be acetone, a more volatile product than the substrate, aminopropane. The temperature can be raised periodically for short periods to flash off the acetone,

25   thereby alleviating equilibrium. Acetone has a boiling point of 47°C, a temperature not likely to degrade the intermediates if used for short periods of time. Most aminotransferases that have activity on alpha-ketoglutarate also have activity on the monatin precursor. Similarly, if a glyoxylate/aromatic acid aminotransferase (EC 2.6.1.60) is used with glycine as the amino donor, glyoxylate is produced which is

30   relatively unstable and has a highly reduced boiling point in comparison to glycine.

EXAMPLE 14: Packet Formulations Comprising Monatin

Packet formulations comprising monatin are prepared to deliver the sweetness of about 2 teaspoons of sugar (approximately 8 grams).

A. Packet formulation with S,S monatin (per gram):

5   787 mg Dextrose

200 mg S,S monatin

10 mg Cream of Tartar

3 mg Calcium Silicate

10   **B. Packet formulation with R,R and S,S monatin (per gram)**

700 mg Dextrose

292 mg Maltodextrin

4 mg R,R monatin

4 mg S, S monatin

15

C. Packet formulation with R,R and S,S monatin (per gram)

992mg agglomerated dextrose with maltodextrin*

4mg R,R monatin

4mg S,S monatin

20

* For example, Unidex® Agglomerated Dextrose 02540 (2034) from CPC International, Inc.

D. Packet formulation with R,R monatin (per gram):

25   500 mg Dextrose

495 mg Maltodextrin

5 mg R,R monatin

E. Half-sized cube with R,R monatin (per gram):

30   999 mg Maltodextrin

1 mg R,R monatin

EXAMPLE 15: Recipes for Packet Formulations Comprising Monatin

The following are examples of how monatin packet formulations, such as those disclosed above, may be used as sugar substitutes in baking and other recipes. The monatin-containing products produced using the following recipes are proposed to be acceptable and of good quality compared to the same products prepared with sugar.

5

Lemonade:

Mix 2 tablespoons of lemon juice and 3 packets (3 g) of a monatin packet formulation with ¾ cup of water in a tall glass until dissolved. Add ice. The monatin-sweetened lemonade will be nearly equivalent in sweetness and equally preferred to the

10   lemonade sweetened with 6 teaspoons (24 g) sucrose and will have significantly fewer calories (about 0 Calories versus 96 Calories).

Whipped Topping

1/3 cup ice water

15   1¼ teaspoon lemon juice

½ teaspoon vanilla

1/3 cup nonfat dry milk powder

3 packets monatin sugar substitute (per Example C)

20   Combine water, lemon juice and vanilla. Stir in nonfat dry milk powder. Beat 10 minutes or until stiff. Add monatin sugar substitute packets and beat 2 minutes.

Sucrose-free sponge cake data sheet

25	Ingredients	% w/w
	cake flour	20.42
	water	18.62
	whole egg	18.2
	polydextrose	17.23
30	High Ratio Shortening	13.44
	Sorbitol Powder	8.62
	Skimmed Milk Powder	1.66
	Baking Powder	1.24
	Salt	0.31
35	R,R Monatin (to desired taste)	0.010-0.040

For every 100 g of cake, 10-40 mg of R,R monatin are used (provided by, for example, 3-10 packets of tabletop monatin sweetener).

EXAMPLE 16: Ready-to-use Formulations Comprising Monatin

"Spoon for spoon" ready-to-use formulations are prepared to be used in place of
5   granulated sugar. Because monatin formulations may have the same volume of sugar,
one may add these formulations straight to beverages or food, or use them directly in
baking as a direct and equal substitute for granulated sugar.

A. Ready-to-use formulation with S,S monatin (per gram):
10   188 mg Hydrolyzed Starch

800 mg Maltodextrin

10 mg S,S monatin

2 mg Sodium Gluconate

B. Ready-to-use formulation with R,R monatin (per gram):
15   999.5 mg Maltodextrin

0.5 mg R,R monatin

C. Ready-to-use formulation with S,S/R,R blend (per gram):
20   972.5 mg Maltodextrin

20 mg Cream of Tartar

4 mg Calcium Silicate

3.1 mg S,S monatin

0.4 mg R,R monatin
25

EXAMPLE 17: Recipes for Ready-to-use Formulations Comprising Monatin

The following are examples of how monatin "spoon for spoon" ready-to-use
formulations, such as those disclosed above, may be used as sugar substitutes in
30   baking and other recipes. The monatin-containing products produced using the
following recipes are proposed to be acceptable and of good quality compared to the
same products prepared with sugar.

French Silk Pie

<u>Crust</u>:

1 cup crushed graham crackers

1 cup chopped pecans

1 stick butter, melted

5

Mix crust ingredients and pat into 9" pie plate. Bake at 300° for 30 minutes.

<u>Filling</u>:

2 squares unsweetened chocolate

10   2 sticks butter

1½ cups monatin ready-to-use formulation (per Example 16B)

2 teaspoons vanilla

4 pasteurized eggs

15   Melt the unsweetened chocolate and cool to room temperature. Cream butter and

monatin sugar substitute. Add vanilla and melted chocolate to the creamed mixture.

Add eggs one at a time beating each egg 4 minutes. Pour over cool crust and

refrigerate overnight.

20   *Blueberry Muffins*:

Note: Sugar can be important in baked goods for texture, browning, as well as

sweetening. In this formula, a small amount of honey is used to compensate for the

lack of sugar in the formula and promote browning and good texture.

25   2 cups all-purpose flour

2 tsp baking powder

¾ tsp salt

½ cup light margarine, softened

1 cup monatin ready-to-use formulation

30   ¼ cup honey

2 whole large eggs

1 tsp vanilla

½ cup skim milk

1 cup blueberries, fresh or frozen

Preheat oven to 350°F. Line 10 muffin cups with paper liners. Sift together flour,
baking powder, salt; set aside. Beat together margarine, monatin sweetener and

5 honey with an electric mixer until light and fluffy. Add eggs one at a time beating
well after each addition. Stir in vanilla. Alternately stir in flour mixture and milk,
beginning and ending with flour mixture. Fold in berries. Spoon batter into paper
lined muffin cups, and bake until golden brown and a toothpick inserted in middle
comes out clean, about 25-30 minutes. Cool in pan 10 minutes on wire rack. Remove

10 muffins from pan. Cool completely on wire rack. Makes 10 muffins.

Strawberry Banana Smoothie:

1 cup orange juice

1 cup fat-free plain yogurt

15 1 frozen banana

1 cup frozen strawberries

6 packets monatin formulation or ¼ cup monatin ready-to-use formulation

Place all items in a blender and blend until smooth. Makes 2 servings. This smoothie

20 has about 33% fewer Calories than the same formula prepared with ¼ cup sugar

Pineapple Orange Sherbet

1 15¼-ounce can crushed pineapple

1 6-ounce can frozen orange juice concentrate, thawed

25 3 cups skim milk

¾ cup evaporated skim milk

1/3 cup monatin ready-to-use formulation (per Example B)

½ teaspoon vanilla

30 Stir together skim milk, undrained pineapple, evaporated skim milk, orange juice
concentrate, monatin sugar substitute and vanilla in a large bowl until the monatin
sugar substitute dissolves. Freeze in 4- or 5-quart ice cream freezer according to
manufacturer's directions. Makes 15 (½ cup) servings.

Lemon Mousse

1 12-ounce can evaporated milk

2 Tablespoons grated lemon rind

Juice from 2 lemons

5   1 cup monatin ready-to-use formulation (per Example A)

12 graham crackers, crushed

Pour milk on jelly roll pan and place in freezer until a sliver of ice forms, about 2

hours. Transfer milk to chilled bowl and whip. Add lemon rind and juice slowly

10   while mixing. Add monatin sugar substitute and continue whipping until soft peaks

form. Transfer to serving dish(es) and sprinkle with graham cracker crumbs. May be

served chilled or frozen.

EXAMPLE 18: Dessert and Confections Products Comprising Monatin

15

Ready-to-use monatin compositions may also be used commercially in the production

of consumer packaged goods.

Instant chocolate pudding data sheet

20

Ingredients	% w/w
Cold Swelling Starch (Ultratex 4)	5.00
Skimmed Milk Powder	14.00
Cocoa Powder	2.5
Maltodextrin	3.5
Chocolate flavoring	0.4
Xanthan Gum	0.20
Salt	0.15
Lecithin Powder	0.25
Disodium Phosphate	0.17
R,R Monatin (to desired taste)	0.005-0.010
Water to	100

For every 100 g of pudding, this corresponds to 5-10 mg of R,R monatin, (provided

35   by, for example, 2-3 packets of tabletop monatin sweetener).

Sugar-free chocolate coating

Ingredients
40   4.0-10% milk solids

15.0-20% chocolate flavoring or chocolate liquors
0-10% cocoa butter
0-2% cocoa
5-10% mannitol

5   0.3-0.5% lecithin
12-25% polydextrose (such as polydextrose K)
0.10-0.5% S,S monatin OR 0.007-0.015% R,R monatin or blends thereof
20-60% vegetable oil

10   For every 100 mg of coating, approximately 2-4 packets of tabletop sweetener are
used.

Sugar-free chocolate

15   Ingredients
2-3 tablespoons cocoa
2 tablespoons butter (no substitutes)
2 tablespoons liquid heavy whipping cream
1/4 teaspoons pure vanilla extract
20   1 tablespoon peanut butter (crunchy or creamy)
6 packets tabletop monatin

EXAMPLE 19: Evaluation of monatin in table-top sweetener applications.

25   Monatin table-top formulations, including R,R monatin or R,R monatin/erythritol
combinations, were assessed relative to other known sweeteners (aspartame and
sucralose) in coffee and iced tea. The key sensory parameters assessed included
sweetness quality, aftertaste, bitter taste and its aftertaste. Qualitative evaluation has
been carried out.

30

Product Formulations

(i)   Coffee

Standard coffee was used in which to evaluate sweetener performance (Table 7).

35   Table 7. Coffee formulation

Ingredient	Supplier	Concentration (%; w/w)	g/700ml
Classic Roast Coffee	Folger®	5.41	37.87
Water		94.59	662.13

Sweeteners were added to coffee at the following concentrations:

Aspartame   0.025% (w/v)
Sucralose   0.0082% (w/v)
R,R monatin*   0.0020, 0.0025, 0.0030% (w/v) plus 1 g maltodextrin
5   R,R monatin/erythritol   0.0020, 0.0025, 0.0030% (w/v) plus 1 g erythritol

(ii)   Iced Tea

An ice tea formulation was developed to evaluate sweetener performance (Table 8).

10

Table 8. Iced Tea formulation

Ingredient	Supplier	Concentration (w/v)
Citric acid		0.200
Sodium citrate		0.020
Tea extract 'Assam' 285002	Plantextrakt	0.150
Natural black tea flavour extract 31108304010000	Rudolph Wild	0.050
Sodium benzoate (20% w/w)		0.075
Sweetener		As required
Water		To volume

Sweeteners were added to tea at the following concentrations:

15   Aspartame   0.0450% (w/v)
Sucralose   0.0170% (w/v)
R,R monatin   0.0030, 0.0035, 0.0040% (w/v) plus 1 g maltodextrin
R,R, monatin/erythritol   0.0030, 0.0035, 0.0040% (w/v) plus 1 g erythritol

20   Sensory Evaluation

The evaluation of these coffee and tea drinks was carried out by a small (n = 6) panel
of experienced sensory evaluators who evaluated the coffee products on one tasting
occasion and the tea products on a subsequent occasion. The results of these
25   evaluations are summarized in Table 9.

Table 9. Sensory evaluation of coffee and tea (200 ml serving size)

Product	Sweetener/concentration	Comments
Coffee	Aspartame/250ppm	Balanced sweetness profile. Very low level of bitterness, probably due to inhibition by APM. Flat, even coffee flavor delivery. Typical APM aftertaste that is perceived at the back of the tongue.
	Sucralose/82ppm	Slow sweetness onset allows stronger coffee notes to be perceived. Bitter coffee notes quite clearly apparent in the aftertaste, although balanced somewhat by the lingering sweet character of sucralose.
	Monatin (25ppm) + Maltodextrin (1 g) (0.5%)	Balanced sweetness profile. Clear coffee flavor in the aftertaste. Stronger coffee flavor overall than with either of the other sweeteners, although this may be (at least in part) due to the limited bitterness inhibiting capacity of monatin.
	Monatin (25ppm) + Erythritol (1 g) (0.5%)	More coffee flavor in monatin sample. Sweetness is less delayed with monatin/erythritol combination than with monatin/maltodextrin. Erythritol smoothes out the coffee flavor and makes the sweetness onset a little faster.
Iced Tea	Aspartame/450ppm	Good temporal characteristics although the typical aspartame flavor is clearly apparent. Balanced, though quite subtle tea flavour. No evidence of flavor enhancement.
	Sucralose/170ppm	Delay in sweetness onset means first impressions are of acidity. Product flavor and overall impression somewhat out of balance because of sweetness profile not matching acidity or flavor profiles.
	Monatin (40ppm) + Maltodextrin (1 g) (0.5%)	Sweetness and flavor profiles very balanced. The lemon flavor notes are clearly enhanced over those of the other sweeteners.
	Monatin (40ppm) + Erythritol (1 g) (0.5%)	Sweetness and flavor profiles balanced. Lemon flavor notes even more enhanced than monatin/maltodextrin alone. The astringency in the aftertaste is greatly reduced/eliminated.

5

<u>Discussion</u>

Monatin delivers unexpected performance benefits, including clear sensory benefits, in table-top sweetener formulations. When used in a table-top formulation added to coffee, a clear increase in the level of coffee flavor is perceived. This benefit is

5   further enhanced through addition of low concentrations of erythritol, which are able to balance and round the flavor and to speed up sweetness onset times. In iced-tea, and particularly acidified acid tea (i.e., with lemon), monatin enhances the lemon flavor notes. Again, erythritol blending with monatin confers additional flavor benefits.

10

Monatin delivers improved sensory properties (e.g., less aftertaste, less off-taste), and/or improved solubility and stability characteristics.

Monatin sweetened coffee contains close to 0 Calories, as compared to 32 Calories in

15   coffee sweetened with 2 teaspoons (~8 g) of sucrose.

EXAMPLE 20: Sweetness dose response curve of monatin and saccharin

Sweetness of monatin and saccharine was assessed using 20 trained sensory

20   evaluators, making judgements in duplicate. Test and reference solutions were prepared in citric/citrate buffer at pH 3.2. *See* FIG. 16. The more linear response of R,R/S,S monatin, as compared to saccharin, is consistent with the delivery of a more sugar-like taste character. The plateau above 10% SEV indicates absence/low levels of "mixture-suppressing" off-tastes and aftertastes. The shape of monatin's dose-

25   response curve is similar to those of aspartame, sucralose and alitame, all of which are "quality" sweeteners.

With R,R/S,S monatin as a sole sweetener in the model system (pH 3.2), the following characteristics were observed: (1) slight delay in sweet taste onset; (2)

30   sweet taste decay was quite rapid; (3) slight "aspartame-like" aftertaste, slightly sweet aftertaste, no bitterness in the aftertaste; and (4) residual cooling sensation in un-flavored systems.

EXAMPLE 21: Stability of monatin at pH 3 with increasing temperatures

A sample of synthetic monatin was subjected to pH 3 at temperatures of 25°C, 50°C and 100°C. At room temperature and pH 3, a 14% loss in monatin was observed over a period of 48 hours. This loss was attributed to lactone formation. At 50°C and pH

5   3, a 23% loss in monatin was observed over a period of 48 hours. This loss was attributed to lactone formation and the buildup of an unknown compound after about 15.5 minutes. At 100°C and pH 3, nearly all monatin was lost after 24 hours. The major detectable component was an unknown at 15.5 minutes.

10   **EXAMPLE 22: Sensory stability of monatin and aspartame at pH 2.5, 3.0, 4.0 at 40°C**

The sensory stability of monatin solutions prepared at pH 2.5, 3.0 and 4.0 and stored at 40°C was monitored for 100 days. Loss of sweetness from these solutions was

15   compared with the losses of sweetness from aspartame solutions prepared and stored under identical conditions.

The sensory stability of monatin (8% SEV, ~55 ppm, synthetic blend containing approximately 96% of the 2R,4R/2S, 4S enantiometric pair and 4% of the

20   2R,4S/2S,4R enantiometric pair) in phosphate/citrate buffers having a pH of 2.5, 3.0, and 4.0 was examined after storage at 40°C. The stability of monatin was compared to that of aspartame (400 ppm) in the same buffers. Three sucrose reference solutions were prepared in the same phosphate/citrate buffers as the monatin and aspartame solutions. All prepared solutions were stored in the dark.

25

Buffer compositions: pH2.5 Phosphoric acid (75% solution) 0.127% (w/v)
                              Tri-sodium citrate monohydrate 0.005% (w/v)
                      pH3.0 Phosphoric acid (75% solution) 0.092% (w/v)
                              Tri-sodium citrate monohydrate 0.031% (w/v)
30   pH4.0 Phosphoric acid (75% solution) 0.071% (w/v)
                              Tri-sodium citrate monohydrate 0.047% (w/v)

The sweetness of each sweetener relative to sucrose was assessed in duplicate by a small panel (n = 8) of trained sensory evaluators experienced in the sweetness

estimation procedure. All samples (in the same buffers) were served in duplicate at a temperature of 22°C ± 1°C. Monatin (test) solutions, coded with 3 digit random number codes were presented individually to panelists, in random order. Sucrose reference standards, ranging from 4.0 – 10.0% (w/v) sucrose, increasing in steps of

5   0.5% (w/v) sucrose were also provided. Panelists were asked to estimate sweetness by comparing the sweetness of the test solution to the sucrose standards. This was carried out by taking 3 sips of the test solution, followed by a sip of water, followed by 3 sips of sucrose standard followed by a sip of water, etc. Panelists were encouraged to estimate the sweetness to one decimal place, e.g., 6.8, 8.5. A five

10   minute rest period was imposed between evaluating the test solutions. Panelists were also asked to rinse well and eat a cracker to reduce any potential carry over effects.

Tables 10 and 11 present results of the stability studies in the phosphate citrate buffers. At each pH and after 100 days' storage at 40°C in the dark, the percentage

15   retention of monatin sweetness was greater than that retained with aspartame. At pH 4.0, the loss of sweetness of the monatin solution appeared almost to have stabilized since there was very little change in measured sweetness intensity between Days 17 and 100, whereas the aspartame solution continued to lose sweetness.

TABLE 10

Sensory Stability of Monatin: Sweetness after 100 Days Storage at 40°C

A.

pH	Time (days)	SEV Monatin (% sucrose)	Retention of Monatin Sweetness (%)	SEV Aspartame (% sucrose)	Retention of Aspartame Sweetness (%)
2.5	0	7.35		7.34	
	1	6.86	93.3	6.90	94.0
	2	6.70	91.2	6.80	92.6
	3	6.50	88.4	6.60	89.9
	4	6.26	85.2	6.29	85.7
	7	6.08	82.7	6.01	81.9
	8	5.98	81.4	5.98	81.5
	9	5.89	80.1	5.97	81.3
	11	5.78	78.6	5.86	79.8
	50	4.61	62.7	4.19	57.1
	100	2.10	28.6	0.80	10.9

B.

pH	Time (days)	SEV Monatin (% sucrose)	Retention of Monatin Sweetness (%)	SEV Aspartame (% sucrose)	Retention of Aspartame Sweetness (%)
3.0	0	7.08		7.15	
	1	7.05	99.6	6.90	96.5
	2	6.60	93.2	6.87	96.1
	3	6.47	91.4	6.60	92.3
	4	6.49	91.6	6.43	89.9
	7	6.04	85.3	6.17	86.3
	8	5.93	83.8	5.93	82.9
	9	5.88	83.1	5.94	83.1
	11	5.88	83.1	5.83	81.5
	50	5.12	72.3	4.71	65.9
	100	4.10	57.9	2.20	30.8

C.

pH	Time (days)	SEV Monatin (% sucrose)	Retention of Monatin Sweetness (%)	SEV Aspartame (% sucrose)	Retention of Aspartame Sweetness (%)
4.0	0	7.40		7.10	
	3	7.08	95.7	6.75	95.1
	8	6.42	86.8	6.23	87.8
	11	6.36	85.9	6.02	84.8
	17	6.10	82.4	5.75	81.0
	24	6.25	84.5	5.85	82.4
	50	6.14	82.9	5.29	74.5
	100	5.80	78.4	4.10	57.7

5   TABLE 11

Stability: Amount of sweetness remaining after 100 days storage at stated pH at 40°C

pH	Sweetener	Sweetness Retained (%)
2.5	Aspartame	11
2.5	Monatin	29
3.0	Aspartame	31
3.0	Monatin	58
4.0	Aspartame	58
4.0	Monatin	78

10

The respective buffers were effective at maintaining pH, as seen in Table 12:

TABLE 12

Sweetener	Nominal pH	Actual pH (after 50 days)
Monatin	2.5	2.39
	3.0	3.13
	4.0	4.28
Aspartame	2.5	2.49
	3.0	3.13
	4.0	4.19

If a *pseudo*-first order breakdown reaction is assumed, a plot of $\log_n$ percentage retention versus time ($\log_n\%RTN$ v. t) allows estimation of the half-life ($t^{1}/_{2}$) and rate constant (k) of sweetness loss under any given set of conditions. In so doing, the kinetics of monatin and aspartame sweetness loss may be summarized as follows in Table 13.

TABLE 13

Sweetener	PH	Half-life ($t^{1}/_{2}$; days)	Rate constant (k; day^{-1})
Monatin	2.5	65 days	$0.011 day^{-1}$
	3.0	115 days	$0.006 day^{-1}$
	4.0	230 days	$0.003 day^{-1}$
Aspartame	2.5	55 days	$0.013 day^{-1}$
	3.0	75 days	$0.009 day^{-1}$
	4.0	140 days	$0.005 day^{-1}$

At each pH and after 100 days storage at 40°C, the percentage retention of monatin sweetness is greater than that retained from aspartame. At pH 4.0, the loss of sweetness of the monatin solution appears almost to have stabilized since there has been very little change in measured sweetness intensity between Days 17 and 100,

5   whereas the aspartame solution continues to lose sweetness.

Estimates of the half-life of monatin and aspartame indicate that sweetness derived from monatin is lost at a slower rate than that from aspartame. Half-life estimates for monatin sweetness at pH 2.5, 3.0 and 4.0 were 65 days, 115 days and 230 days,

10   respectively. Aspartame half-life estimates were 55 days, 75 days and 140 days under the same conditions.

Thus, under acidic conditions and storage at 40°C, monatin delivers a more stable sweetness than does aspartame.

15

EXAMPLE 23: Production of erythritol/ monatin granules

A solution of 2000 g erythritol, 16 g R,R monatin and 5 L water is prepared in a small tank at 40°C. 58 kg of erythritol is put into the basket of a fluid bed. The air

20   temperature of the fluid bed is set at 65°C. The monatin/erythritol solution is sprayed on the fluid bed at 25kg/hr for 17 minutes. Additional heating time of 20 minutes is required to dry the powder. Product is sieved over a 1250 mm sieve. *See also* EP 0 325 790 B1 (Mitsubishi, Nikken 1993). The calculated relative sweetness of the monatin/erythritol product (as compared to sucrose) is approximately 1.10. Triangle

25   tests are performed with two monatin/erythritol samples and one monatin/maltodextrin sample. Triangle tests are known in the art. It is expected that ready-to-use cubes prepared with monatin/erythritol will have a color and crystalline quality that closely resembles sucrose.

30   **EXAMPLE 24: Chromatography of stereoisomers of monatin**

Sample Preparation – Approximately 50-75 µg of lyophilized material was placed in a microcentrifuge tube. To this 1.0 mL of HPLC grade methanol was added. The

solution was vortexed for 30 minutes, centrifuged and an aliquot of the supernatant was removed for analysis.

Reversed Phase HPLC – Chromatography of two distinct diastereomer peaks

5   (R,R/S,S and R,S/S,R) was accomplished using a 2.1 x 250mm Xterra™ MS C$_8$ 5μm (Waters Corporation) HPLC column. Detection was carried out using an Ultima™ triple quadrupole mass spectrometer from Micromass. Mobile phase was delivered by the following gradient:

10

Time (min)		0	9	16	20	21
0.05%TFA	A%	95	65	10	10	95
Methanol, 0.05% TFA	B%	5	35	90	90	5
Flow mL/min		0.25	0.25	0.25	0.25	0.25

15   *Chiral HPLC* – Chromatography of two distinct monatin stereoisomers (R,R and S,S) was accomplished using a 250 x 4.6 mm Chirobiotic T(Advanced Separations Technologies, Inc.) HPLC column. Detection was carried out using an Ultima™ triple quadrupole mass spectrometer from Micromass. Mobile phase consisted of Methanol with 0.2% Acetic acid and 0.05% ammonium hydroxide.

20

Mass Spectrometry (MS/MS) – The presence of monatin was detected by a Selected Reaction Monitoring (SRM) experiment. The protonated molecular ion of monatin ([M+H]$^+$) has a m/z=293.3. Fragmentation of this molecular ion produces a significant ion at m/z=257.3 arising from multiple dehydrations of the molecular ion.

25   This transition has been shown to be very specific to monatin and was chosen as the transition (293.3 to 257.3) for monitoring during the SRM experiment. This method of detection was employed for both reversed phase and chiral separations of monatin.

Results – The standard samples of R,S/S,R and S,S/R,R were evaluated under

30   Reversed Phase HPLC. The samples were prepared by derivatization and enzymatic resolution. Chromatograms for standard solutions are displayed in FIG. 17. Following the reversed phase analysis, chiral chromatography was performed to evaluate specific isomers present in the samples. Chiral chromatography of standard S,S and R,R, monatin solutions are displayed in FIG. 18.

35

EXAMPLE 25: Stability of monatin at high temperature (80 °C) and neutral pH

A 100 milliliter solution of 75 ppm monatin at pH 7 was used as a stock solution. The synthetic monatin sample contained approximately 96% of the 2R,4R/2S,4S

5   enantiomeric pair and 4% of the 2R,4S/2S,4R enantiomeric pair. Samples were incubated at 80°C and pH 7 for the duration of the experiment and samples were withdrawn at 0, 1, 2, 3, 4 hours and 1, 2, 4, 7, 14, 21 and 35 days. All experimental conditions were run in duplicate.

10   *Separation and Quantification Using LC-MS using Reverse Phase Chromatography* – A response curve was established for both detected diastereomer peaks of the synthetic monatin. A range of 5-150 ppm was bracketed with the synthetic monatin standard dissolved in DI water. Separation of the two diastereomer peaks was accomplished using a 3.9 x 150mm Novapak C18 (Waters Corporation) HPLC

15   column. Ultraviolet-Visible (UV) and Mass Spectrometer (MS) detectors were used in series for detection and quantitation. Monatin and its lactone peak each have a UV_{max} at 279 nm that aided in precise detection. Quantification was done by acquiring Selected Ion Monitoring (SIM) scan of 293.3 m/z and 275.3 m/z in positive-ion electrospray mode.

20

Results – At a neutral pH (common for baking and desserts), the degree of degradation of monatin was determined to be insignificant even after 7-35 days. The disappearance of monatin over time is highly dependent on pH since the primary byproducts are cyclization and possibly very small levels of racemization. During the

25   experiment at 80°C and pH 7, no change in concentration of racemic RR/SS monatin or lactones thereof was detected within the limits of precision afforded by using LC-MS for quantitation. Based on this data, it is expected that at high temperatures and neutral pH, monatin stability is acceptable for baking.

30

In view of the many possible embodiments to which the principles of this disclosure may be applied, it should be recognized that the illustrated embodiments are only particular examples of the disclosure and should not be taken as a limitation on the scope of the disclosure. For example, from the teachings of the disclosure herein, it

would be apparant to a person of ordinary skill that a monatin sweetener compostion can be made in liquid concentrate form, rather than in a solid form, wherein a chosen volume of the liquid concentrate, e.g., 1 mL or 0.35 mL, would have a sweetness comparable to a chosen amount, such as two teaspoons, of granulated sugar.

5

Claims:

1.   A tabletop sweetener composition comprising monatin or salt thereof, wherein the composition provides a sweetness comparable to about 0.9 to about 9.0 grams of granulated sugar.

2.   The tabletop sweetener composition of claim 1, wherein a 1 gram portion of the composition provides a sweetness comparable to two teaspoons of granulated sugar.

3.   The tabletop sweetener composition of claim 1, wherein 1 gram of the composition contains less calories and carbohydrates than about 1 gram of granulated sugar.

4.   The tabletop sweetener composition of claim 1, wherein 1 gram of the composition comprises from about 0 to about 200 mg S,S monatin or salt thereof, and from about 0 to about 5 mg R,R monatin or salt thereof.

5.   The tabletop sweetener composition of claim 1, wherein 1 gram of the composition comprises from about 3 to about 200 mg S,S monatin or salt thereof, and from about 0 to about 5 mg R,R monatin or salt thereof.

6.   The tabletop sweetener composition of claim 1, wherein 1 gram of the composition comprises from about 0 to about 200 mg S,S monatin or salt thereof, and from about 3 to about 5 mg R,R monatin or salt thereof.

7.   The tabletop sweetener composition of claim 1, wherein 1 gram of the composition comprises about 200 mg or less S,S monatin or salt thereof, and wherein the monatin or salt thereof is substantially free of R,R, S,R or R,S monatin or salt thereof.

8.   The tabletop sweetener composition of claim 1, wherein 1 gram of the composition comprises about 5 mg or less R,R monatin or salt thereof, and wherein the monatin or salt thereof is substantially free of S,S, S,R or R,S monatin or salt thereof.

9.   The tabletop sweetener composition of claim 1, further comprising at least one ingredient chosen from bulking agents, carriers, fibers, sugar alcohols, oligosaccharides, sugars, non-monatin high intensity sweeteners, nutritive sweeteners, flavorings, flavor enhancers, flavor stablizers, acidulants, anti-caking agents, and free-flow agents.

10.   The tabletop sweetener composition of claim 1, further comprising erythritol.

11.   The tabletop sweetener composition of claim 10, wherein the composition comprises up to 99.7% erythritol.

12.   The tabletop sweetener composition of claim 1, further comprising trehalose.

13.   The tabletop sweetener composition of claim 1, further comprising cyclamate.

14.   The tabletop sweetener composition of claim 1, further comprising agglomerated dextrose with maltodextrin.

15.   The tabletop sweetener composition of claim 1, wherein the monatin or salt thereof consists essentially of R,R monatin or salt thereof.

16.   The tabletop sweetener composition of claim 1, wherein the monatin or salt thereof consists essentially of S,S monatin or salt thereof.

17.   The tabletop sweetener composition of claim 1, wherein the monatin or salt thereof is a stereoisomerically-enriched R,R monatin or salt thereof.

18.   The tabletop sweetener composition of claim 1, wherein the monatin or salt thereof is a stereoisomerically-enriched S,S monatin or salt thereof.

19.   The tabletop sweetener composition of claim 17, wherein the monatin or salt thereof comprises at least 95% R,R monatin or salt thereof.

20.   The tabletop sweetener composition of claim 2, wherein the sweetness is provided by monatin or salt thereof produced in a biosynthetic pathway.

21.   A ready-to-use sweetener composition comprising monatin or salt thereof, wherein a volume of the composition provides a sweetness comparable to a same volume of granulated sugar.

22.   The ready-to-use sweetener composition of claim 21, wherein 1 teaspoon of the composition contains less calories and carbohydrates than about 1 teaspoon of granulated sugar.

23.   The ready-to-use sweetener composition of claim 21, wherein 1 gram of the composition comprises about 3 to about 25 mg S,S monatin or salt thereof, and from about 0 to about 0.625 mg R,R monatin or salt thereof

24.   The ready-to-use sweetener composition of claim 21, wherein the monatin or salt thereof consists essentially of S,S monatin or salt thereof or R,R monatin or salt thereof.

25.   The ready-to-use sweetener composition of claim 21, wherein 1 gram of the composition comprises from about 5 to about 25 mg S,S monatin or salt thereof.

26.   The ready-to-use sweetener composition of claim 21, wherein 1 gram of the composition comprises from about 0.4 to about 0.625 R,R monatin or salt thereof, and wherein the monatin or salt thereof is substantially free of S,S, S,R or R,S monatin or salt thereof.

27.   The ready-to-use sweetener composition of claim 21, wherein 1 gram of the composition comprises from about 0.5 to about 1 mg R,R monatin or salt thereof, and wherein the monatin or salt thereof is substantially free of S,S, S,R or R,S monatin or salt thereof.

28.   The ready-to-use sweetener composition of claim 21, further comprising erythritol.

29.   The ready-to-use sweetener composition of claim 28, wherein the composition comprises up to 99.7% erythritol.

30.   The ready-to-use sweetener composition of claim 21, further comprising trehalose.

31.   The ready-to-use sweetener composition of claim 21, further comprising cyclamate.

32.   A sweetener composition comprising a stereoisomerically-enriched monatin mixture, wherein the monatin mixture is produced via a biosynthetic pathway.

33.   The sweetener composition of claim 32, wherein the biosynthetic pathway is a multi-step pathway and at least one step of the multi-step pathway is a chemical conversion.

34.   The sweetener composition of claim 32, wherein the mixture is predominantly R,R monatin or salt thereof.

35.   A homogeneous tabletop sweetener composition comprising monatin or salt thereof, wherein a sample of the composition comprises monatin or salt thereof in an amount ranging from more than 2 mg to about 200 mg, and wherein the monatin or salt thereof in the sample provides a sweetness comparable to about 0.9 to about 9.0 grams of granulated sugar.

36.   The composition of claim 35, wherein the monatin or salt thereof in the sample provides a sweetness comparable to two teaspoons of granulated sugar.

37.   The composition of claim 35, wherein the sample weighs about 1 gram.

38.   The composition of claim 35, wherein the sample comprises an amount of monatin or salt thereof ranging from more than 2 mg to about 5 mg monatin or salt thereof.

39.   The composition of claim 38, wherein the composition is substantially free of S,S monatin or salt thereof.

40.   The tabletop sweetener of claim 35, wherein the composition is substantially free of R,R monatin or salt thereof.

41.   A tabletop sweetener composition comprising monatin or salt thereof, wherein a sample of the composition comprises monatin or salt thereof in an amount ranging from more than 2 mg to about 105 mg monatin or salt thereof, and wherein the monatin or salt thereof in the sample provides a sweetness comparable to one teaspoon of granulated sugar.

42.   The tabletop sweetener of claim 35 or 41, wherein the sample has a volume of about 0.35 ml.

43.   The tabletop sweetener of claim 35 or 41, wherein the sample is a cube of granulated material.

44.   A tabletop sweetener composition comprising a monatin composition produced in a biosynthetic pathway, and wherein the monatin composition does not contain petrochemical, toxic or hazardous contaminants.

45.   A tabletop sweetener composition comprising monatin or salt thereof, wherein the monatin or salt thereof is produced in a biosynthetic pathway and isolated from a recombinant cell, and wherein the recombinant cell does not contain petrochemical, toxic or hazardous contaminants.

46.   A method for making a sweetener composition comprising monatin or salt thereof, wherein the method comprises producing monatin or salt thereof from at least one substrate chosen from glucose, tryptophan, indole-3-lactic acid, indole-3-pyruvate and the monatin precursor.

47.   The method of claim 46, wherein the method further comprises combining the monatin or salt thereof with erythritol.

48.   The method of claim 46, wherein the method further comprises combining the monatin or salt thereof with trehalose.

49.   The method of claim 46, wherein the method further comprises combining the monatin or salt thereof with cyclamate.

50.   The method of claim 46, wherein the method further comprises combining the monatin or salt thereof with at least one other ingredient that is not monatin or salt thereof.

51.   The method of claim 50, wherein the at least one other ingredient is chosen from bulking agents, carriers, fibers, sugar alcohols, oligosaccharides, sugars, non-monatin high intensity sweeteners, nutritive sweeteners, flavorings, flavor enhancers, flavor stabilizers, acidulants, anti-caking, free-flow agents, and any combination thereof.

52.   The method of claim 50, wherein the at least one other ingredient is chosen from maltodextrin, dextrose, erythritol and fiber.

53.   The method of claim 50, wherein a portion of the sweetener composition weighing about 1 gram comprises from about 0 mg to about 200 mg S,S monatin or salt thereof and from about 0 mg to about 5 mg R,R monatin or salt thereof, and wherein the portion provides a sweetness comparable to two teaspoons of granulated sugar.

54.   The method of claim 50, wherein 1 gram of the sweetener composition comprises from about 0 to about 25 mg S,S monatin or salt thereof and from about 0 to about 0.625 mg R,R monatin or salt thereof, and wherein a volume of the composition has a sweetness comparable to a same volume of granulated sugar.

55.   The method of claim 50, wherein 1 gram of the composition comprises from about 0 to about 25 mg S,S monatin or salt thereof and from about 0 to about 0.625 mg R,R monatin or salt thereof, and wherein 1 gram of the composition has a sweetness comparable to about 0.9 to about 9.0 grams granulated sugar.

56.   The method of claim 53, wherein the amount of S,S monatin or salt thereof ranges from about 5 to about 200 mg S,S monatin or salt thereof per 1 gram of the composition.

57.   The method of claim 56, wherein the method further comprises combining S,S monatin or salt thereof with at least one other ingredient chosen from bulking agents, carriers, fibers, sugar alcohols, oligosaccharides, sugars, non-monatin high intensity sweeteners, nutritive sweeteners, flavorings, flavor enhancers, flavor stablizers, acidulants, anti-caking, free-flow agents, and any combination thereof, wherein the monatin or salt thereof comprises about 3 to about 200 mg of monatin or salt thereof per 1 gram of the composition.

58.   The method of claim 53, wherein the method further comprises combining R,R monatin or salt thereof with at least one other ingredient chosen from bulking agents, carriers, fibers, sugar alcohols, oligosaccharides, sugars, non-monatin high intensity sweeteners, nutritive sweeteners, flavorings, flavor enhancers, flavor stablizers, acidulants, anti-caking, free-flow agents, and any combination thereof, wherein the amount of R,R monatin or salt thereof ranges from about 3 to about 5 mg of R,R monatin or salt thereof per 1 gram of the composition.

59.   The method of claim 50, wherein the monatin or salt thereof comprises from about 0.4 to about 5 mg R,R monatin or salt thereof per 1 gram of the composition.

60.   The method of claim 46, wherein the method further comprises combining the monatin or salt thereof with at least one bulking agent chosen from dextrose and maltodextrin, and wherein the monatin or salt thereof comprises about 5 mg R,R monatin or salt thereof per 1 gram of the composition.

61.   The method of claim 46, wherein the method further comprises combining the monatin or salt thereof with maltodextrin, and wherein the monatin or salt thereof comprises from about 0.5 to about 1 mg R,R monatin or salt thereof per 1 gram of the composition.

62.   A method for making a sweetener composition comprising a monatin composition, wherein the method comprises: (a) producing monatin or salt thereof in a biosynthetic pathway in a recombinant cell; (b) isolating the monatin composition from the recombinant cell, wherein the monatin composition consists of monatin or salt thereof and other edible or potable material.

63.   A monatin composition comprising monatin and erythritol.

1/14

Glucose

+ serine

Tryptophan   Indole-3-lactic acid

PLP and amino acceptor
or NAD(P)
or H_2O and O_2 and FAD
                                    NAD(P)
                                    or H_2O and O_2 and FMN

Indole-3-pyruvate

+ C3 molecule

±PLP

2-hydroxy 2-(indol-3-ylmethyl)-4-keto
glutaric acid (MP)

PLP and amino donor

or NAD(P)H + NH3

Monatin

FIG. 1

```
                    ┌─────────────────┐
                    │    Glucose      │
                    └─────────────────┘
                             │        U.S. Patent No. 4,371,614
                             ▼
                    ┌─────────────────┐
                    │   Tryptophan    │
                    └─────────────────┘
```

EC 2.6.1.27 tryptophan aminotransferase
EC 2.6.1.5 tyrosine (aromatic) aminotransferase
EC 1.4.1.19 tryptophan dehydrogenase
EC 1.4.1.2-4 glutamate dehydrogenase
EC 1.4.1.20 phenylalanine dehydrogenase
EC 2.6.1.28 tryptophan-phenylpyruvate transaminase
EC 2.6.1.- multiple substrate aminotransferase
EC 2.6.1.1 aspartate aminotransferase
EC 1.4.3.2 L-amino acid oxidase
tryptophan oxidase (no EC number)
D-tryptophan aminotransferase (no EC number)
EC 1.4.99.1 D-amino acid dehydrogenase
EC 1.4.3.3 D-amino acid oxidase
EC 2.6.1.21 D-alanine aminotransferase

```
          ┌──────────────────────┐
          │   Indole-3-pyruvate  │
          └──────────────────────┘
```

EC 4.1.3.-, 4.1.2.- lyase, synthase

EC 4.1.3.16 4-Hydroxy-2-oxoglutarate glyoxylate-lyase

EC 4.1.3.17 4-Hydroxy-4-methyl-2-oxoglutarate pyruvate-lyase

Chemical aldol condensation

```
┌──────────────────────────────────────────────────────────────┐
│  2-hydroxy 2-(indol-3-ylmethyl)-4-keto glutaric acid           │
└──────────────────────────────────────────────────────────────┘
```

EC 2.6.1.27 tryptophan aminotransferase
EC 2.6.1.5 tyrosine (aromatic) aminotransferase
EC 1.4.1.19 tryptophan dehydrogenase
EC 2.6.1.28 tryptophan-phenylpyruvate transaminase
EC 2.6.1.- multiple substrate aminotransferase
EC 2.6.1.1 aspartate aminotransferase
EC 1.4.1.2-4 glutamate dehydrogenase
EC 1.4.1.20 phenylalanine dehydrogenase
EC 1.4.99.1 D-amino acid dehydrogenase
EC 2.6.1.21 D-alanine aminotransferase
D-tryptophan aminotransferase (no EC number)

```
          ┌─────────────────┐
          │    Monatin      │        FIG. 2
          └─────────────────┘
```

FIG. 3

Indole-3-lactic acid

EC 1.1.1.110 indolelactate dehydrogenase

EC 1.1.1.222 R-4-hydroxyphenyllactate dehydrogenase

EC 1.1.1.237 3-(4)-hydroxyphenylpyruvate reductase

EC 1.1.1.27, 1.1.1.28, 1.1.2.3 lactate dehydrogenase

EC 1.1.1.111 (3-imidazol-5-yl) lactate dehydrogenase

EC 1.1.3.- lactate oxidase

Chemical oxidation

Indole-3-pyruvate

FIG. 4

Enolate chemistry
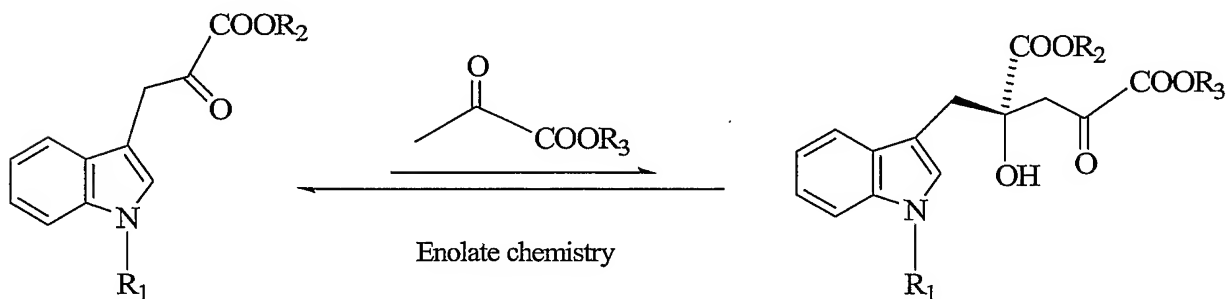
R_1 = Boc, Cbz, etc.

R_2 and R_3 = Alkyl, Aryl, etc.

FIG. 5A

03-Apr-2002
13:52:54

TIC

FIG. 5B

m/z = 293

Time (min)

FIG. 6

FIG. 7A

08-Apr-2002
15:11:01
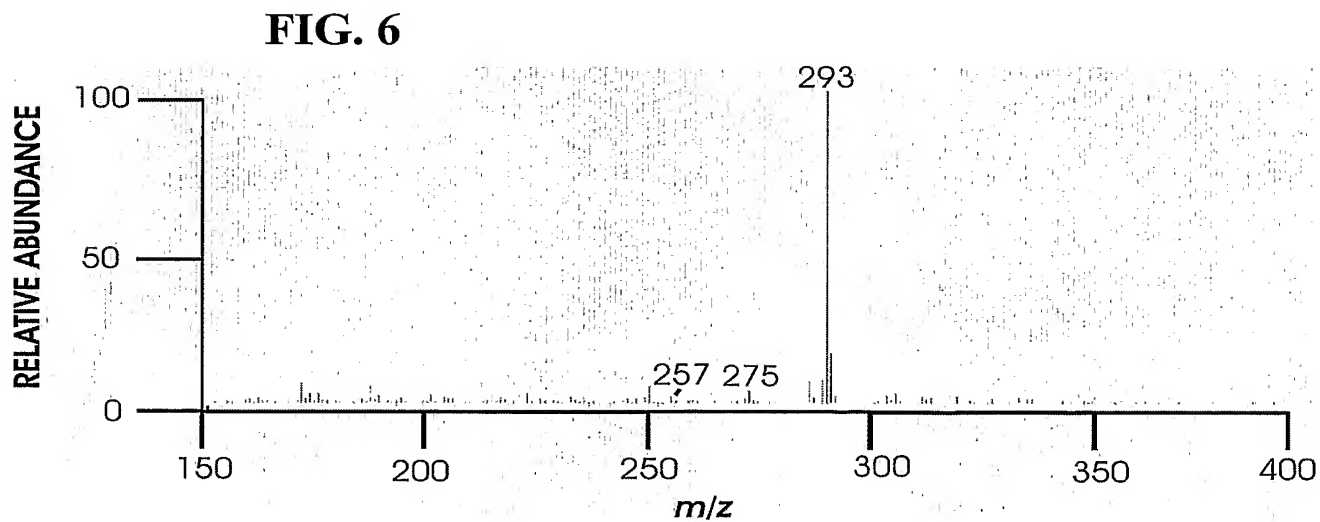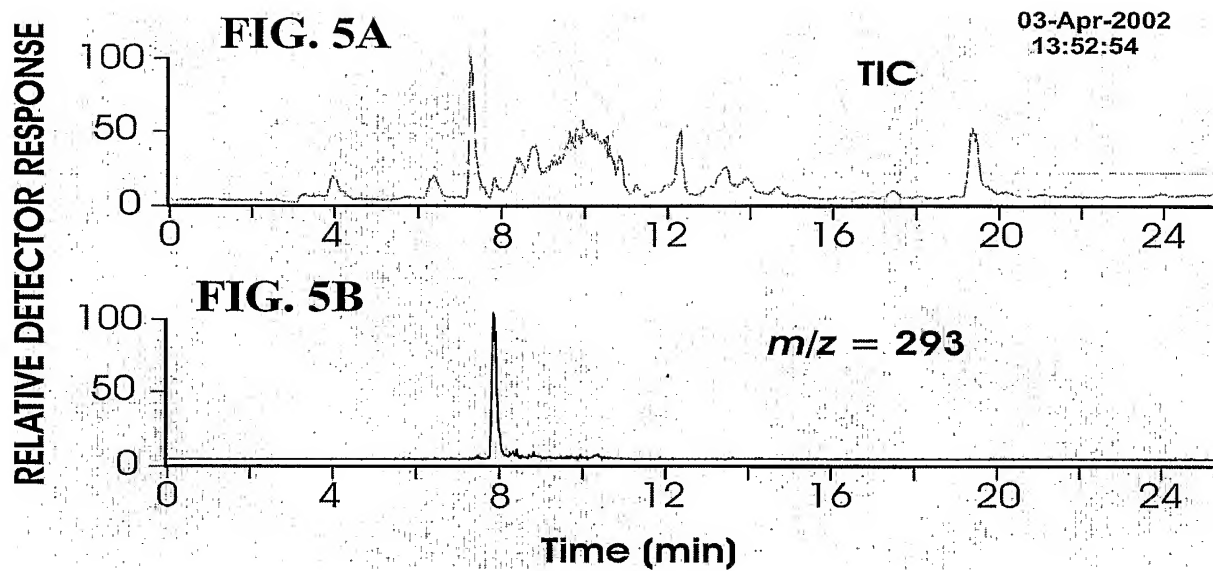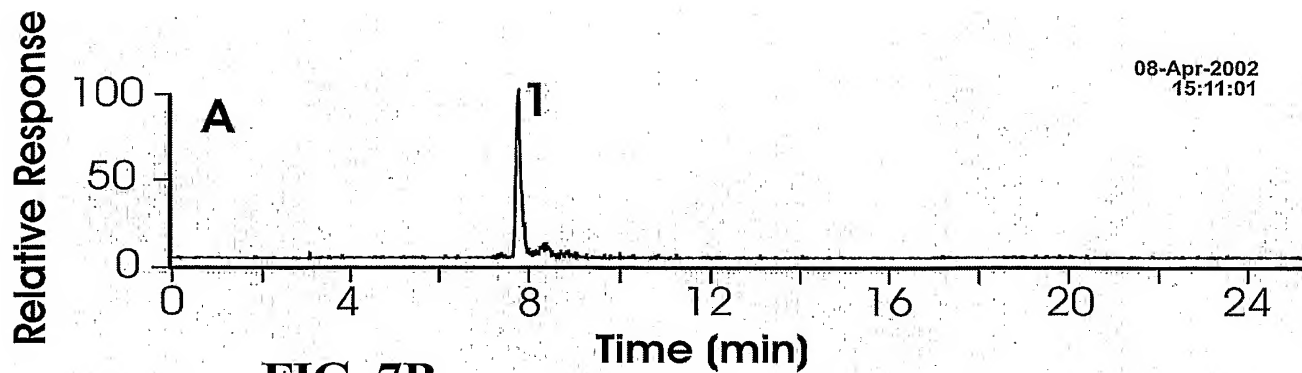
FIG. 7B
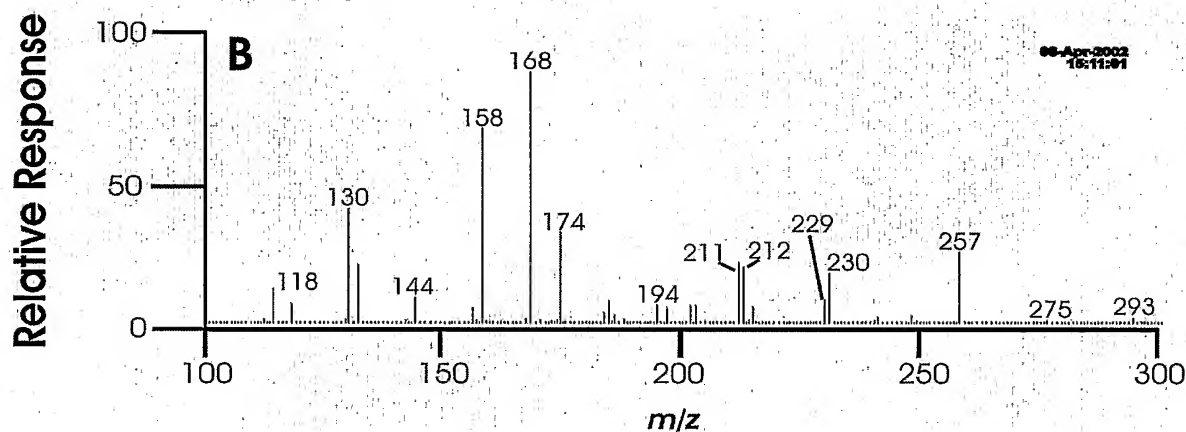
FIG. 8

+TOF MS: 124 MCA scans from Sample 7 (293jjd_with Tryptophan) of car0709a.wif...   Max. 9.3e4 counts.
a=3.56970808537750280e-004, t0=-1.24626879588351580e+002 R;

VB342   10-Jul-2002
                                                      13:11:33
071002202a   1: Scan ES+
100   205
                                                     1.21e8

FIG. 9A

%

0

071002201a   1: Scan ES+
100   205
                                                     1.24e8

FIG. 9B

%

0

071002203a   1: Scan ES+
100   293
                                                     1.02e8

FIG. 9C

%

0

                2.00   4.00   6.00   8.00   10.00 12.00 14.00 16.00 18.00 20.00 22.00 24.00   Time

FIG. 10

FIG. 11

FIG. 12

tryptophan

tryptophan oxidase or O_2
amino acid oxidase H_2O_2 *catalase*

indole-3-pyruvate

pyruvate   *ProA aldolase*

monatin precursor (MP)

lysine
ε-aminotransferase   lysine
allysine

monatin   *spontaneous, - H20*

1-Piperideine 6-carboxylate

FIG. 13

FIG. 14:

FIG. 15

Sensory Procedure:

Sweetness matching using 20 trained
assessors making judgements in duplicate

Test and reference solutions prepared in
citric/citrate buffer at pH 3.2

FIG. 16

FIG. 17

FIG. 18

SEQUENCE LISTING

<110> Cargill, Incorporated

<120> Polypeptides and Biosynthetic Pathways

<130> 6682-64349

<150> 60/374,831
<151> 2002-04-23

<160> 74

<170> PatentIn version 3.1

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<211> 1170
<212> DNA
<213> Sinorhizobium meliloti

<400> 1
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ggacgcacgc cgatcttccg ggccgtcaag gcggcggaaa agcggcttct cgaaacacag   180

gacagcaagg cctatatcgg ccccgaaggg gacctcgtct ttctcgatcg gctctgggaa   240

ctcgtcggcg gcgacacgat cgagcggagc catgttgcgg gcgtccagac gcccggcggc   300

tccggcgcgc tccgtttggc ggcggacctc atcgcccgca tgggcggccg aggcatctgg   360

ctcgggctgc cgagctggcc gaaccacgcg ccgatcttca aggcggccgg gctcgatatc   420

gccacctacg acttcttcga cattccgtcg cagtcggtca tcttcgataa tctggtgagc   480

gcgctggaag cgccgcatc cggcgatgcg gtgctgctgc atgcaagctg ccacaacccg   540

accggcggcg tcctgagcga agcacaatgg atggagatcg ccgcgctggt ggccgagcgc   600

ggcctgctgc cgctcgtcga tctcgcctat caggggttcg ccgcggcct cgaccaggat   660

gtcgcgggcc tccggcatct tctcggcgtg gtcccggaag cgctcgtcgc ggtttcctgc   720

tcgaagtcct tcgggcttta tcgcgagcgc gcgggcgcga tcttcgcgcg gaccagctcg   780

actgcctcgg cggacagggt gcgctcaaac ctcgcgggcc tcgcacgcac cagctattcc   840

atgccgccgg atcacggcgc agccgtcgtg cggacgatcc ttgacgaccc ggaactcagg   900

cgcgactgga cggaggagct cgagacgatg cggctcagga tgacgggcct ccggcggtcg   960

cttgccgagg gactccgcac ccgctggcag agcctcggcg cagtcgccga tcaggagggc   1020

atgttctcca tgctgccgct ttccgaagcg gaggttatgc ggctcaggac cgagcacggc   1080

atctatatgc cggcatccgg ccgcatcaac atcgccgggc tgaagacggc ggaagccgcc   1140

gagattgccg gcaagttcac cagtctctga   1170

<210> 2
<211> 389
<212> PRT
<213> Sinorhizobium meliloti

<400> 2

Met Phe Asp Ala Leu Ala Arg Gln Ala Asp Asp Pro Leu Leu Phe Leu
1   5   10   15

Ile Gly Leu Phe Arg Lys Asp Glu Arg Pro Gly Lys Val Asp Leu Gly
                20   25   30

Val Gly Val Tyr Arg Asp Glu Thr Gly Arg Thr Pro Ile Phe Arg Ala
            35   40   45

Val Lys Ala Ala Glu Lys Arg Leu Leu Glu Thr Gln Asp Ser Lys Ala
    50   55   60

Tyr Ile Gly Pro Glu Gly Asp Leu Val Phe Leu Asp Arg Leu Trp Glu
65   70   75   80

Leu Val Gly Gly Asp Thr Ile Glu Arg Ser His Val Ala Gly Val Gln
                85   90   95

Thr Pro Gly Gly Ser Gly Ala Leu Arg Leu Ala Ala Asp Leu Ile Ala
            100   105   110

Arg Met Gly Gly Arg Gly Ile Trp Leu Gly Leu Pro Ser Trp Pro Asn
        115   120   125

His Ala Pro Ile Phe Lys Ala Ala Gly Leu Asp Ile Ala Thr Tyr Asp
    130   135   140

Phe Phe Asp Ile Pro Ser Gln Ser Val Ile Phe Asp Asn Leu Val Ser
145   150   155   160

Ala Leu Glu Gly Ala Ala Ser Gly Asp Ala Val Leu Leu His Ala Ser
                165   170   175

Cys His Asn Pro Thr Gly Gly Val Leu Ser Glu Ala Gln Trp Met Glu
                180   185   190

Ile Ala Ala Leu Val Ala Glu Arg Gly Leu Leu Pro Leu Val Asp Leu
        195   200   205

```
Ala Tyr Gln Gly Phe Gly Arg Gly Leu Asp Gln Asp Val Ala Gly Leu
    210             215         220
```

```
Arg His Leu Leu Gly Val Val Pro Glu Ala Leu Val Ala Val Ser Cys
225             230         235                 240
```

```
Ser Lys Ser Phe Gly Leu Tyr Arg Glu Arg Ala Gly Ala Ile Phe Ala
            245             250             255
```

```
Arg Thr Ser Ser Thr Ala Ser Ala Asp Arg Val Arg Ser Asn Leu Ala
        260             265             270
```

```
Gly Leu Ala Arg Thr Ser Tyr Ser Met Pro Pro Asp His Gly Ala Ala
        275             280             285
```

```
Val Val Arg Thr Ile Leu Asp Asp Pro Glu Leu Arg Arg Asp Trp Thr
    290             295             300
```

```
Glu Glu Leu Glu Thr Met Arg Leu Arg Met Thr Gly Leu Arg Arg Ser
305             310             315                 320
```

```
Leu Ala Glu Gly Leu Arg Thr Arg Trp Gln Ser Leu Gly Ala Val Ala
            325             330             335
```

```
Asp Gln Glu Gly Met Phe Ser Met Leu Pro Leu Ser Glu Ala Glu Val
            340             345             350
```

```
Met Arg Leu Arg Thr Glu His Gly Ile Tyr Met Pro Ala Ser Gly Arg
        355             360             365
```

```
Ile Asn Ile Ala Gly Leu Lys Thr Ala Glu Ala Ala Glu Ile Ala Gly
    370             375             380
```

```
Lys Phe Thr Ser Leu
385
```

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<210>   3
<211>   1260
<212>   DNA
<213>   Rhodobacter sphaeroides

<400>   3
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aaggatgacg aaggaatgct gaccgccctg aagccgcagc ccgcggacaa gatcctgcaa     120

ctgatccaga tgttccgcga ggatgcgcgc gcggacaaga tcgatctggg cgtgggcgtc     180

tacaaggacc cgaccgggct caccccggtc atgcgggccg tgaaggcggc cgagaagcgg     240
```

```
ctctgggagg tcgagaccac caagacctac accggccttg ccgacgagcc ggcctacaat    300

gccgcgatgg cgaagctgat cctcgcgggc gcggtcccgg ccgaccgggt ggcctcggtc    360

gccaccgggc gcggcacggg cgcggtgcgt caggcgctcg agctgatccg catggcctcg    420

cccgaggcca ccgtctggat ctcgaacccg acctggccga accatctgtc gatcgtgaaa    480

tatctcggca tcccgatgcg ggaataccgc tatttcgacg ccgagaccgg cgccgtcgat    540

gccgagggca tgatggagga tctggcccag gtgaaggcgg cgacgtggt gctgctgcac    600

ggctgctgcc acaacccgac cggcgccaac ccgaacccgg tgcagtggct ggccatctgc    660

gagagcctgg cccggacagg cgcggtgccg ctgatcgacc tcgcctatca gggcttcggc    720

gacgggctcg agatggatgc ggcggcgacg cggcttctgg ccaccagact gcccgaggtg    780

ctgatcgcgg cctcctgctc gaagaacttc ggcatctacc gcgagcgcac gggcatcctg    840

atcgccatcg cgaggcggc gggccggggc acggtgcagg ccaacctcaa cttcctgaac    900

cggcagaact actccttccc gccggaccat ggcgcgcggc tcgtgaccat gatcctcgag    960

gacgagacgc tgagcgccga ctggaaggcg gaactcgagg aggtgcggct caacatgctg    1020

acactgcgcc gccagcttgc cgatgcgctg caggccgaga ccggctcgaa ccgcttcggc    1080

ttcgtggccg agcatcgcgg catgttctcg cgcctcggga tcacgcccgc cgaggtggag    1140

cggctgcgga ccgagcacgg ggtctacatg gtgggcgatt cgcggctgaa catcgcgggg    1200

ctgaaccgga cgaccgtgcc ggtgctggcg cgcgcggtgg ccaaggtgct gcgcggctga    1260
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<210>  4

<211>  419
<212>  PRT
<213>  Rhodobacter sphaeroides

<400>  4

Met Arg Ser Thr Thr Ala Pro Gly Pro Ser Gly Ala Cys Met Thr Ile
1               5                   10                  15


Ser Arg Ser Arg Lys Asp Asp Glu Gly Met Leu Thr Ala Leu Lys Pro
            20                  25                  30


Gln Pro Ala Asp Lys Ile Leu Gln Leu Ile Gln Met Phe Arg Glu Asp
        35                  40                  45


Ala Arg Ala Asp Lys Ile Asp Leu Gly Val Gly Val Tyr Lys Asp Pro
    50                  55                  60


Thr Gly Leu Thr Pro Val Met Arg Ala Val Lys Ala Ala Glu Lys Arg
```

65	70	75	80

Leu Trp Glu Val Glu Thr Thr Lys Thr Tyr Thr Gly Leu Ala Asp Glu
                85   90   95

Pro Ala Tyr Asn Ala Ala Met Ala Lys Leu Ile Leu Ala Gly Ala Val
            100   105   110

Pro Ala Asp Arg Val Ala Ser Val Ala Thr Pro Gly Gly Thr Gly Ala
            115   120   125

Val Arg Gln Ala Leu Glu Leu Ile Arg Met Ala Ser Pro Glu Ala Thr
130   135   140

Val Trp Ile Ser Asn Pro Thr Trp Pro Asn His Leu Ser Ile Val Lys
145   150   155   160

Tyr Leu Gly Ile Pro Met Arg Glu Tyr Arg Tyr Phe Asp Ala Glu Thr
                165   170   175

Gly Ala Val Asp Ala Glu Gly Met Met Glu Asp Leu Ala Gln Val Lys
            180   185   190

Ala Gly Asp Val Val Leu Leu His Gly Cys Cys His Asn Pro Thr Gly
            195   200   205

Ala Asn Pro Asn Pro Val Gln Trp Leu Ala Ile Cys Glu Ser Leu Ala
            210   215   220

Arg Thr Gly Ala Val Pro Leu Ile Asp Leu Ala Tyr Gln Gly Phe Gly
225   230   235   240

Asp Gly Leu Glu Met Asp Ala Ala Ala Thr Arg Leu Leu Ala Thr Arg
                245   250   255

Leu Pro Glu Val Leu Ile Ala Ala Ser Cys Ser Lys Asn Phe Gly Ile
            260   265   270

Tyr Arg Glu Arg Thr Gly Ile Leu Ile Ala Ile Gly Glu Ala Ala Gly
            275   280   285

Arg Gly Thr Val Gln Ala Asn Leu Asn Phe Leu Asn Arg Gln Asn Tyr
            290   295   300

Ser Phe Pro Pro Asp His Gly Ala Arg Leu Val Thr Met Ile Leu Glu
305   310   315   320

Asp Glu Thr Leu Ser Ala Asp Trp Lys Ala Glu Leu Glu Glu Val Arg
             325   330   335

Leu Asn Met Leu Thr Leu Arg Arg Gln Leu Ala Asp Ala Leu Gln Ala
             340   345   350

Glu Thr Gly Ser Asn Arg Phe Gly Phe Val Ala Glu His Arg Gly Met
             355   360   365

Phe Ser Arg Leu Gly Ile Thr Pro Ala Glu Val Glu Arg Leu Arg Thr
     370   375   380

Glu His Gly Val Tyr Met Val Gly Asp Ser Arg Leu Asn Ile Ala Gly
385   390   395   400

Leu Asn Arg Thr Thr Val Pro Val Leu Ala Arg Ala Val Ala Lys Val
             405   410   415

Leu Arg Gly

<210>   5
<211>   1260
<212>   DNA
<213>   Rhodobacter sphaeroides

<400>   5
atgcgctcta cgacggctcc tggtccgagt ggggcatgta tgacgatctc aaggtcgcga   60

aaggatgacg aaggaatgct gaccgccctg aagccgcagc ccgcggacaa gatcctgcaa   120

ctgatccaga tgttccgcga ggatgcgcgc gcggacaaga tcgatctggg cgtgggcgtc   180

tacaaggacc cgaccgggct cacccccggtc atgcgggccg tgaaggccgc cgagaagcgg   240

ctctgggagg tcgagaccac caagacctac accggccttg ccggcgagcc cgcctacaat   300

gccgcgatgg cgaagctgat cctcgcaggc gcggtcccgg ccgaccgggt ggcctcggtc   360

gccacccccg gcggcacggg cgcggtgcgt caggcgctcg agctgatccg catggcctcg   420

cccgaggcca ctgtctggat ctcgaacccg acctggccga accatctgtc gatcgtgaaa   480

tatctcggca tcccgatgcg ggaataccgc tatttcgacg ccgagaccgg cgccgtcgat   540

gccgagggct tgatggagga tctggcccag gtgaaggcgg cgacgtggt gctgctgcac   600

ggctgctgcc acaacccgac cggcgccaac ccgaacccgg tgcagtggct ggccgtctgc   660

gagagcctgg cccggacagg cgcggtgccg ctgatcgacc tcgcctatca gggcttcggc   720

gacgggctcg agatggatgc ggcggcgacg cggcttctgg ccaccagact gcccgaggtg   780

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ctgatcgcgg cctcctgctc gaagaacttc ggcatctacc gcgagcgaac gggcatcctg      840

atcgccatcg gcgaggcggc gggccggggc acggtgcagg ccaacctcaa cttcctgaac      900

cggcagaact actccttccc gccggaccat ggcgcgcggc tcgtgaccat gatcctcgag      960

gacgagacgc tgagcgccga ctggaaggcg gaactcgagg aggtgcggct caacatgctg      1020

acgctgcgcc gccagcttgc cgatgcgctg caggccgaga ccggctcgaa ccgcttcggc      1080

ttcgtggccg agcatcgcgg catgttctcg cgcctcggga tcacgcccgc cgaggtggag      1140

cggctgcgga ccgagcacgg ggtctacatg gtgggcgatt cgcggctgaa catcgcgggg      1200

ctgaaccgga cgaccgtgcc ggtgctggcg cgcgcggtgg ccaaggtgct cgcgcggctga     1260
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<210>   6
<211>   419
<212>   PRT
<213>   Rhodobacter sphaeroides

<400>   6
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```
Met Arg Ser Thr Thr Ala Pro Gly Pro Ser Gly Ala Cys Met Thr Ile
1               5                   10                  15


Ser Arg Ser Arg Lys Asp Asp Glu Gly Met Leu Thr Ala Leu Lys Pro
            20                  25                  30


Gln Pro Ala Asp Lys Ile Leu Gln Leu Ile Gln Met Phe Arg Glu Asp
        35                  40                  45


Ala Arg Ala Asp Lys Ile Asp Leu Gly Val Gly Val Tyr Lys Asp Pro
    50                  55                  60


Thr Gly Leu Thr Pro Val Met Arg Ala Val Lys Ala Ala Glu Lys Arg
65                  70                  75                  80


Leu Trp Glu Val Glu Thr Thr Lys Thr Tyr Thr Gly Leu Ala Gly Glu
                85                  90                  95


Pro Ala Tyr Asn Ala Ala Met Ala Lys Leu Ile Leu Ala Gly Ala Val
            100                 105                 110


Pro Ala Asp Arg Val Ala Ser Val Ala Thr Pro Gly Gly Thr Gly Ala
            115                 120                 125


Val Arg Gln Ala Leu Glu Leu Ile Arg Met Ala Ser Pro Glu Ala Thr
    130                 135                 140
```

Val Trp Ile Ser Asn Pro Thr Trp Pro Asn His Leu Ser Ile Val Lys
145   150   155   160

Tyr Leu Gly Ile Pro Met Arg Glu Tyr Arg Tyr Phe Asp Ala Glu Thr
                  165   170   175

Gly Ala Val Asp Ala Glu Gly Leu Met Glu Asp Leu Ala Gln Val Lys
              180   185   190

Ala Gly Asp Val Val Leu Leu His Gly Cys Cys His Asn Pro Thr Gly
          195   200   205

Ala Asn Pro Asn Pro Val Gln Trp Leu Ala Val Cys Glu Ser Leu Ala
      210   215   220

Arg Thr Gly Ala Val Pro Leu Ile Asp Leu Ala Tyr Gln Gly Phe Gly
225   230   235   240

Asp Gly Leu Glu Met Asp Ala Ala Ala Thr Arg Leu Leu Ala Thr Arg
              245   250   255

Leu Pro Glu Val Leu Ile Ala Ala Ser Cys Ser Lys Asn Phe Gly Ile
              260   265   270

Tyr Arg Glu Arg Thr Gly Ile Leu Ile Ala Ile Gly Glu Ala Ala Gly
          275   280   285

Arg Gly Thr Val Gln Ala Asn Leu Asn Phe Leu Asn Arg Gln Asn Tyr
      290   295   300

Ser Phe Pro Pro Asp His Gly Ala Arg Leu Val Thr Met Ile Leu Glu
305   310   315   320

Asp Glu Thr Leu Ser Ala Asp Trp Lys Ala Glu Leu Glu Glu Val Arg
              325   330   335

Leu Asn Met Leu Thr Leu Arg Arg Gln Leu Ala Asp Ala Leu Gln Ala
          340   345   350

Glu Thr Gly Ser Asn Arg Phe Gly Phe Val Ala Glu His Arg Gly Met
          355   360   365

Phe Ser Arg Leu Gly Ile Thr Pro Ala Glu Val Glu Arg Leu Arg Thr
      370   375   380

Glu His Gly Val Tyr Met Val Gly Asp Ser Arg Leu Asn Ile Ala Gly

385   390   395   400

Leu Asn Arg Thr Thr Val Pro Val Leu Ala Arg Ala Val Ala Lys Val
                    405   410   415

Leu Arg Gly

<210> 7
<211> 1239
<212> DNA
<213> Leishmania major

<400> 7
atgtccatgc aggcggccat gaccacggcg gagcgctggc agaagattca ggcacaagct   60

cccgatgtca tcttcgatct cgcaaaacgc gccgccgctg ccaagggccc caaggccaac   120

ctcgtcattg gtgcctaccg cgacgagcag ggccgtccct atccgctacg cgtggtccgc   180

aaggctgagc agcttctctt ggacatgaat ctcgactacg agtacctacc catctcgggc   240

taccagccct tcatcgatga ggcggtaaag attatctacg gcaataccgt cgagctggag   300

aacctggttg cggtgcagac gctgagcggg accggtgctg tctctctcgg ggcgaagctg   360

ctgactcgcg tcttcgacgc tgagacgacg cccatctacc tttccgaccc cacgtggccc   420

aaccactacg gcgtcgtgaa ggctgctggc tggaagaaca tctgcacgta cgcctactac   480

gaccccaaga cggtcagcct gaatttcgag ggcatgaaga aagacattct ggcggcgccg   540

gacggctccg tgttcattct gcaccagtgc gcgcacaacc ccaccggcgt ggacccgtcg   600

caggagcagt ggaacgagat cgcgtcactg atgctggcca agcaccatca ggtgttcttc   660

gactccgcct accaaggcta tgcgagcggc agcctcgaca cggacgcgta tgctgcccgc   720

ctgtttgccc gccgcggcat cgaggtactg ctggcgcagt cgttctccaa gaacatgggc   780

ttgtacagcg agcgtgcagg cacgctgtcg ctgctcctca aggacaagac gaagcgcgcg   840

gatgtaaaga gcgtgatgga ttcgctgatc cgtgaggagt acacgtgccc cccagcccac   900

ggtgcccgct agcccacct aatcctgagc aacaacgaac tgcgaaagga gtgggaggca   960

gagctatcag ccatggcaga gcgcatccgt acgatgcgcc gcaccgtgta cgacgagctg   1020

ctgcgcctgc agacgcccgg gagctgggaa catgtcatta ccagattgg catgtttttcc   1080

ttcctcgggc tgtcaaaggc gcagtgcgaa tactgccaaa accacaacat cttcatcaca   1140

gtgtcgggcc gcgctaacat ggcaggtctg acgcatgaga cggcgctgat gctagcacag   1200

acgatcaacg atgctgtgcg caatgtgaat cgtgagtga   1239

<210> 8

<211> 412
<212> PRT
<213> Leishmania major

<400> 8

Met Ser Met Gln Ala Ala Met Thr Thr Ala Glu Arg Trp Gln Lys Ile
1   5   10   15

Gln Ala Gln Ala Pro Asp Val Ile Phe Asp Leu Ala Lys Arg Ala Ala
            20   25   30

Ala Ala Lys Gly Pro Lys Ala Asn Leu Val Ile Gly Ala Tyr Arg Asp
        35   40   45

Glu Gln Gly Arg Pro Tyr Pro Leu Arg Val Val Arg Lys Ala Glu Gln
    50   55   60

Leu Leu Leu Asp Met Asn Leu Asp Tyr Glu Tyr Leu Pro Ile Ser Gly
65   70   75   80

Tyr Gln Pro Phe Ile Asp Glu Ala Val Lys Ile Ile Tyr Gly Asn Thr
                85   90   95

Val Glu Leu Glu Asn Leu Val Ala Val Gln Thr Leu Ser Gly Thr Gly
            100   105   110

Ala Val Ser Leu Gly Ala Lys Leu Leu Thr Arg Val Phe Asp Ala Glu
        115   120   125

Thr Thr Pro Ile Tyr Leu Ser Asp Pro Thr Trp Pro Asn His Tyr Gly
    130   135   140

Val Val Lys Ala Ala Gly Trp Lys Asn Ile Cys Thr Tyr Ala Tyr Tyr
145   150   155   160

Asp Pro Lys Thr Val Ser Leu Asn Phe Glu Gly Met Lys Lys Asp Ile
            165   170   175

Leu Ala Ala Pro Asp Gly Ser Val Phe Ile Leu His Gln Cys Ala His
            180   185   190

Asn Pro Thr Gly Val Asp Pro Ser Gln Glu Gln Trp Asn Glu Ile Ala
        195   200   205

Ser Leu Met Leu Ala Lys His His Gln Val Phe Phe Asp Ser Ala Tyr
    210   215   220

```
Gln Gly Tyr Ala Ser Gly Ser Leu Asp Thr Asp Ala Tyr Ala Ala Arg
225             230             235             240

Leu Phe Ala Arg Arg Gly Ile Glu Val Leu Leu Ala Gln Ser Phe Ser
            245             250             255

Lys Asn Met Gly Leu Tyr Ser Glu Arg Ala Gly Thr Leu Ser Leu Leu
            260             265             270

Leu Lys Asp Lys Thr Lys Arg Ala Asp Val Lys Ser Val Met Asp Ser
        275             280             285

Leu Ile Arg Glu Glu Tyr Thr Cys Pro Pro Ala His Gly Ala Arg Leu
    290             295             300

Ala His Leu Ile Leu Ser Asn Asn Glu Leu Arg Lys Glu Trp Glu Ala
305             310             315             320

Glu Leu Ser Ala Met Ala Glu Arg Ile Arg Thr Met Arg Arg Thr Val
            325             330             335

Tyr Asp Glu Leu Leu Arg Leu Gln Thr Pro Gly Ser Trp Glu His Val
            340             345             350

Ile Asn Gln Ile Gly Met Phe Ser Phe Leu Gly Leu Ser Lys Ala Gln
        355             360             365

Cys Glu Tyr Cys Gln Asn His Asn Ile Phe Ile Thr Val Ser Gly Arg
    370             375             380

Ala Asn Met Ala Gly Leu Thr His Glu Thr Ala Leu Met Leu Ala Gln
385             390             395             400

Thr Ile Asn Asp Ala Val Arg Asn Val Asn Arg Glu
            405             410
```

<210> 9
<211> 1182
<212> DNA
<213> Bacillus subtilis

<400> 9
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tcgaatcttg tagcccaaca cgaagacgtc atttcactta caatcggcca gcctgatttt     120

ttcacaccgc atcatgtgaa agctgccgca aaaaagcca ttgatgaaaa cgtgacgtca     180
```

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tatactccga atgccggcta cctggagctg agacaagctg tgcagcttta tatgaagaaa    240

aaagcggatt tcaactatga tgctgaatct gaaattatca tcacaacagg cgcaagccaa    300

gccattgatg ctgcattccg gacgatttta tctcccggtg atgaagtcat tatgccaggg    360

cctatttatc cgggctatga acctattatc aatttgtgcg gggccaagcc tgtcattgtt    420

gatactacgt cacacggctt taagcttacc gcccggctga ttgaagatgc tctgacaccc    480

aacaccaagt gtgtcgtgct tccttatccg tcaaaccta ccggcgtgac tttatctgaa    540

gaagaactga aaagcatcgc agctctctta aaaggcagaa atgtcttcgt attgtctgat    600

gaaatataca gtgaattaac atatgacaga ccgcattact ccatcgcaac ctatttgcgg    660

gatcaaacga ttgtcattaa cgggttgtca aaatcacaca gcatgaccgg ttggagaatt    720

ggatttttat ttgcaccgaa agacattgca aagcacattt taaaggttca tcaatacaat    780

gtgtcgtgcg cctcatccat ttctcaaaaa gccgcgcttg aagctgtcac aaacggcttt    840

gacgatgcat tgattatgag agaacaatac aaaaaacgtc tggactatgt ttatgaccgt    900

cttgtttcca tgggacttga cgtagttaaa ccgtccggtg cgttttatat cttcccttct    960

attaaatcat ttggaatgac ttcatttgat tttagtatgg ctcttttgga agacgctggc   1020

gtggcactcg tgccgggcag ctcgttctca acatatggtg aaggatatgt aaggctgtct   1080

tttgcatgct caatggacac gctgagagaa ggcctagacc gtttagaatt atttgtatta   1140

aaaaaacgtg aagcaatgca gacgataaac aacggcgttt aa                       1182
```

<210>   10
<211>   393
<212>   PRT
<213>   Bacillus subtilis

<400>   10

```
Met Glu His Leu Leu Asn Pro Lys Ala Arg Glu Ile Glu Ile Ser Gly
1               5                   10                  15


Ile Arg Lys Phe Ser Asn Leu Val Ala Gln His Glu Asp Val Ile Ser
                20                  25                  30


Leu Thr Ile Gly Gln Pro Asp Phe Phe Thr Pro His His Val Lys Ala
            35                  40                  45


Ala Ala Lys Lys Ala Ile Asp Glu Asn Val Thr Ser Tyr Thr Pro Asn
        50                  55                  60


Ala Gly Tyr Leu Glu Leu Arg Gln Ala Val Gln Leu Tyr Met Lys Lys
65                  70                  75                  80
```

Lys Ala Asp Phe Asn Tyr Asp Ala Glu Ser Glu Ile Ile Ile Thr Thr
             85   90   95

Gly Ala Ser Gln Ala Ile Asp Ala Ala Phe Arg Thr Ile Leu Ser Pro
          100   105   110

Gly Asp Glu Val Ile Met Pro Gly Pro Ile Tyr Pro Gly Tyr Glu Pro
          115   120   125

Ile Ile Asn Leu Cys Gly Ala Lys Pro Val Ile Val Asp Thr Thr Ser
    130   135   140

His Gly Phe Lys Leu Thr Ala Arg Leu Ile Glu Asp Ala Leu Thr Pro
145   150   155   160

Asn Thr Lys Cys Val Val Leu Pro Tyr Pro Ser Asn Pro Thr Gly Val
          165   170   175

Thr Leu Ser Glu Glu Glu Leu Lys Ser Ile Ala Ala Leu Leu Lys Gly
          180   185   190

Arg Asn Val Phe Val Leu Ser Asp Glu Ile Tyr Ser Glu Leu Thr Tyr
          195   200   205

Asp Arg Pro His Tyr Ser Ile Ala Thr Tyr Leu Arg Asp Gln Thr Ile
    210   215   220

Val Ile Asn Gly Leu Ser Lys Ser His Ser Met Thr Gly Trp Arg Ile
225   230   235   240

Gly Phe Leu Phe Ala Pro Lys Asp Ile Ala Lys His Ile Leu Lys Val
          245   250   255

His Gln Tyr Asn Val Ser Cys Ala Ser Ser Ile Ser Gln Lys Ala Ala
          260   265   270

Leu Glu Ala Val Thr Asn Gly Phe Asp Asp Ala Leu Ile Met Arg Glu
          275   280   285

Gln Tyr Lys Lys Arg Leu Asp Tyr Val Tyr Asp Arg Leu Val Ser Met
          290   295   300

Gly Leu Asp Val Val Lys Pro Ser Gly Ala Phe Tyr Ile Phe Pro Ser
305   310   315   320

Ile Lys Ser Phe Gly Met Thr Ser Phe Asp Phe Ser Met Ala Leu Leu
            325   330   335

Glu Asp Ala Gly Val Ala Leu Val Pro Gly Ser Ser Phe Ser Thr Tyr
            340   345   350

Gly Glu Gly Tyr Val Arg Leu Ser Phe Ala Cys Ser Met Asp Thr Leu
            355   360   365

Arg Glu Gly Leu Asp Arg Leu Glu Leu Phe Val Leu Lys Lys Arg Glu
    370   375   380

Ala Met Gln Thr Ile Asn Asn Gly Val
385   390

<210> 11
<211> 1176
<212> DNA
<213> Lactobacillus amylovorus

<400> 11
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ggtgaaccag atatgaatac tcctgagcat gttaagcaag cggctattaa gaatattgca   180

gataatgatt cacactatgc tccacaaaag ggaaagcttg aattaagaaa agctatcagt   240

aaatatttga aaagattac tggaattgaa tatgatccag aaacagaaat cgtagtaaca   300

gttggtgcaa ctgaagcaat taacgctacc ttgtttgcta ttactaatcc gggtgacaag   360

gttgcaattc ctacgccagt cttttctcta tattggcccg tggctacact tgctgatgcc   420

gattatgttt tgatgaatac tgcagaagat ggtttttaagt taacacctaa gaagttagaa   480

gaaactatca agaaaatcc aacaattaaa gcagtaattt tgaattatcc aactaaccca   540

actggtgttg aatatagcga agatgaaatt aaagctttgg ctaaggtaat taaagataat   600

catctgtacg taattaccga tgaaatttac agtactttga cttacggtgt aaaacacttt   660

tcaattgcca gcttaattcc agaaagagca atttatatct ctggtttatc taaatcacat   720

gcgatgactg gttatcgttt aggctatgtt gccggacctg caaaaattat ggcagaaatt   780

ggtaaagttc atggccttat ggtgacgact acgacggatt catcacaagc tgccgcaatt   840

gaagcacttg aacacggact tgatgaccct gagaaatata gggaagttta tgaaaagcgt   900

cgtgactatg tttaaagga attagccgag atagagatgc aagcagttaa gccagaaggt   960

gcattttata tctttgctaa aattccagct aagtatggca aagacgatat gaaatttgcc   1020

```
ttggatttag cttttaaaga aaaagtgggt atcactccag gtagtgcatt tggtcctggt   1080

ggtgaaggtc atattagatt atcttatgca tcaagtgatg aaaacttgca tgaggcaatg   1140

aagcgaatga agaaagtttt acaagaggac gaataa                             1176
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<210> 12
<211> 391
<212> PRT
<213> Lactobacillus amylovorus

<400> 12

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Met Pro Glu Leu Ala Asn Asp Leu Gly Leu Ser Lys Lys Ile Thr Asp
1               5                   10                  15


Val Lys Ala Ser Gly Ile Arg Ile Phe Asp Asn Lys Val Ser Ala Ile
            20                  25                  30


Pro Gly Ile Ile Lys Leu Thr Leu Gly Glu Pro Asp Met Asn Thr Pro
        35                  40                  45


Glu His Val Lys Gln Ala Ala Ile Lys Asn Ile Ala Asp Asn Asp Ser
    50                  55                  60


His Tyr Ala Pro Gln Lys Gly Lys Leu Glu Leu Arg Lys Ala Ile Ser
65                  70                  75                  80


Lys Tyr Leu Lys Lys Ile Thr Gly Ile Glu Tyr Asp Pro Glu Thr Glu
                85                  90                  95


Ile Val Val Thr Val Gly Ala Thr Glu Ala Ile Asn Ala Thr Leu Phe
            100                 105                 110


Ala Ile Thr Asn Pro Gly Asp Lys Val Ala Ile Pro Thr Pro Val Phe
        115                 120                 125


Ser Leu Tyr Trp Pro Val Ala Thr Leu Ala Asp Ala Asp Tyr Val Leu
    130                 135                 140


Met Asn Thr Ala Glu Asp Gly Phe Lys Leu Thr Pro Lys Lys Leu Glu
145                 150                 155                 160


Glu Thr Ile Lys Glu Asn Pro Thr Ile Lys Ala Val Ile Leu Asn Tyr
                165                 170                 175


Pro Thr Asn Pro Thr Gly Val Glu Tyr Ser Glu Asp Glu Ile Lys Ala
            180                 185                 190
```

```
Leu Ala Lys Val Ile Lys Asp Asn His Leu Tyr Val Ile Thr Asp Glu
        195             200             205


Ile Tyr Ser Thr Leu Thr Tyr Gly Val Lys His Phe Ser Ile Ala Ser
        210             215             220


Leu Ile Pro Glu Arg Ala Ile Tyr Ile Ser Gly Leu Ser Lys Ser His
225             230             235             240


Ala Met Thr Gly Tyr Arg Leu Gly Tyr Val Ala Gly Pro Ala Lys Ile
                245             250             255


Met Ala Glu Ile Gly Lys Val His Gly Leu Met Val Thr Thr Thr Thr
                260             265             270


Asp Ser Ser Gln Ala Ala Ala Ile Glu Ala Leu Glu His Gly Leu Asp
        275             280             285


Asp Pro Glu Lys Tyr Arg Glu Val Tyr Glu Lys Arg Arg Asp Tyr Val
        290             295             300


Leu Lys Glu Leu Ala Glu Ile Glu Met Gln Ala Val Lys Pro Glu Gly
305             310             315             320


Ala Phe Tyr Ile Phe Ala Lys Ile Pro Ala Lys Tyr Gly Lys Asp Asp
                325             330             335


Met Lys Phe Ala Leu Asp Leu Ala Phe Lys Glu Lys Val Gly Ile Thr
                340             345             350


Pro Gly Ser Ala Phe Gly Pro Gly Gly Glu Gly His Ile Arg Leu Ser
        355             360             365


Tyr Ala Ser Ser Asp Glu Asn Leu His Glu Ala Met Lys Arg Met Lys
        370             375             380


Lys Val Leu Gln Glu Asp Glu
385             390
```

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<210>   13
<211>   1413
<212>   DNA
<213>   R. sphaeroides

<400>   13
atgcgcgagc ctcttgccct cgagatcgac ccgggccacg gcggcccgct gttcctcgcc        60
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atcgccgagg cgatcaccct cgacatcacc cgcgggcggc tgaggcccgg agcgagactg    120

cccggcacac gcgcgctggc gcgggcgctc ggcgtgcatc gcaacacggt ggatgccgcc    180

tatcaggagt tgctgaccca gggctggctg caggccgagc ccgcgcgggg caccttcgtg    240

gcgcaggatc tgccgcaggg gatgctggtg cacaggcccg cgcccgcgcc ggtcgagccg    300

gtcgcgatgc gcgcgggggct cgccttctcc gatggcgcgc cggaccccga gctggtgccc    360

gacaaggcgc tggcgcgggc ctttcgccgg gcgctcctgt cgcccgcctt ccgcgccgga    420

gcggattacg gcgatgcccg cggcacctcc tcgctgcggg aggcgctggc agcctatctc    480

gcctcggacc ggggcgtggt cgcggatcct gcgcggctgc tgatcgcgcg gggcagccag    540

atggcgctgt cctggtagc ccgggcggcg ctggcgccgg agaggcgat cgcggtcgag    600

gagccgggct atccgctggc ctgggaggcg ttccgcgcag cgggagcgga ggtgcgcggc    660

gtgccggtgg atggcggcgg cctcaggatc gacgcgctcg aggccgcgct ggcccgggat    720

ccgcgaatcc gggcggtcta tgtcacgccc catcaccagt atccgacgac cgtcaccatg    780

ggcgcggcgc ggcggttgca gcttctggaa ctggcagagc gccaccggct cgcgctgatc    840

gaggacgact acgaccacga ataccgcttc gagggccgtc cggtgctgcc gctggctgcc    900

cgcgcgccgg aaggtctgcc gctgatctat gtgggctcgc tgtcgaaact gctctcgccc    960

ggtatccggc tgggatacgc gctggcgccc gagcggctgc tgacccgcat ggccgcggcg   1020

cgcgccgcca tcgaccggca gggcgacgcg ccgctcgagg cggcgctggc cgagctgatc   1080

cgcgacggcg atctgggccg tcatgcccgc aaggcgcgca gggtctaccg ggcgcggcgg   1140

gatctgctgg cggagcgtct cacggcgcag ctggccgggc gcgccgcctt cgatctgccg   1200

gccggggggcc tcgcgctgtg gctgcgctgc gcgggcgtct cggccgagac ctgggccgaa   1260

gccgcagggc aggcgggggct cgccctgctg ccgggcacgc gcttcgcgct ggagagcccg   1320

gcgccgcagg ccttccggct gggctatgcg gcgctggacg aggggcagat cgcccgggcg   1380

gtggagatcc tcgcccggag cttccccggc tga    1413
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<210> 14
<211> 470
<212> PRT
<213> R. sphaeroides

<400> 14

```
Met Arg Glu Pro Leu Ala Leu Glu Ile Asp Pro Gly His Gly Gly Pro
1               5                   10                  15


Leu Phe Leu Ala Ile Ala Glu Ala Ile Thr Leu Asp Ile Thr Arg Gly
            20                  25                  30
```

Arg Leu Arg Pro Gly Ala Arg Leu Pro Gly Thr Arg Ala Leu Ala Arg
    35   40   45

Ala Leu Gly Val His Arg Asn Thr Val Asp Ala Ala Tyr Gln Glu Leu
    50   55   60

Leu Thr Gln Gly Trp Leu Gln Ala Glu Pro Ala Arg Gly Thr Phe Val
65   70   75   80

Ala Gln Asp Leu Pro Gln Gly Met Leu Val His Arg Pro Ala Pro Ala
            85   90   95

Pro Val Glu Pro Val Ala Met Arg Ala Gly Leu Ala Phe Ser Asp Gly
            100   105   110

Ala Pro Asp Pro Glu Leu Val Pro Asp Lys Ala Leu Ala Arg Ala Phe
        115   120   125

Arg Arg Ala Leu Leu Ser Pro Ala Phe Arg Ala Gly Ala Asp Tyr Gly
    130   135   140

Asp Ala Arg Gly Thr Ser Ser Leu Arg Glu Ala Leu Ala Ala Tyr Leu
145   150   155   160

Ala Ser Asp Arg Gly Val Val Ala Asp Pro Ala Arg Leu Leu Ile Ala
            165   170   175

Arg Gly Ser Gln Met Ala Leu Phe Leu Val Ala Arg Ala Ala Leu Ala
            180   185   190

Pro Gly Glu Ala Ile Ala Val Glu Glu Pro Gly Tyr Pro Leu Ala Trp
            195   200   205

Glu Ala Phe Arg Ala Ala Gly Ala Glu Val Arg Gly Val Pro Val Asp
    210   215   220

Gly Gly Gly Leu Arg Ile Asp Ala Leu Glu Ala Ala Leu Ala Arg Asp
225   230   235   240

Pro Arg Ile Arg Ala Val Tyr Val Thr Pro His His Gln Tyr Pro Thr
            245   250   255

Thr Val Thr Met Gly Ala Ala Arg Arg Leu Gln Leu Leu Glu Leu Ala
            260   265   270

```
Glu Arg His Arg Leu Ala Leu Ile Glu Asp Asp Tyr Asp His Glu Tyr
    275             280             285

Arg Phe Glu Gly Arg Pro Val Leu Pro Leu Ala Ala Arg Ala Pro Glu
    290             295             300

Gly Leu Pro Leu Ile Tyr Val Gly Ser Leu Ser Lys Leu Leu Ser Pro
305             310             315             320

Gly Ile Arg Leu Gly Tyr Ala Leu Ala Pro Glu Arg Leu Leu Thr Arg
            325             330             335

Met Ala Ala Ala Arg Ala Ala Ile Asp Arg Gln Gly Asp Ala Pro Leu
            340             345             350

Glu Ala Ala Leu Ala Glu Leu Ile Arg Asp Gly Asp Leu Gly Arg His
            355             360             365

Ala Arg Lys Ala Arg Arg Val Tyr Arg Ala Arg Arg Asp Leu Leu Ala
    370             375             380

Glu Arg Leu Thr Ala Gln Leu Ala Gly Arg Ala Ala Phe Asp Leu Pro
385             390             395             400

Ala Gly Gly Leu Ala Leu Trp Leu Arg Cys Ala Gly Val Ser Ala Glu
            405             410             415

Thr Trp Ala Glu Ala Ala Gly Gln Ala Gly Leu Ala Leu Leu Pro Gly
            420             425             430

Thr Arg Phe Ala Leu Glu Ser Pro Ala Pro Gln Ala Phe Arg Leu Gly
            435             440             445

Tyr Ala Ala Leu Asp Glu Gly Gln Ile Ala Arg Ala Val Glu Ile Leu
    450             455             460

Ala Arg Ser Phe Pro Gly
465             470
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<210>   15
<211>   35
<212>   DNA
<213>   Artificial sequence

<220>
<223>   primer
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<400> 15
ggtattgagg gtcgcatgaa ggttttagtc aatgg   35

<210> 16
<211> 37
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 16
agaggagagt tagagcctta tgaaatgcta gcagcct   37

<210> 17
<211> 35
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 17
ggtattgagg gtcgcatgtt cgacgccctc gcccg   35

<210> 18
<211> 37
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 18
agaggagagt tagagcctca gagactggtg aacttgc   37

<210> 19
<211> 35
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 19
ggtattgagg gtcgcatgga acatttgctg aatcc   35

<210> 20
<211> 37
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 20

agaggagagt tagagcctta aacgccgttg tttatcg   37

<210> 21
<211> 35
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 21
ggtattgagg gtcgcatgcg cgagcctctt gccct   35

<210> 22
<211> 37
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 22
agaggagagt tagagcctca gccggggaag ctccggg   37

<210> 23
<211> 35
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 23
ggtattgagg gtcgcatgtc cacgcaggcg gccat   35

<210> 24
<211> 37
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 24
agaggagagt tagagcctca ctcacgattc acattgc   37

<210> 25
<211> 35
<212> DNA
<213> Artificial sequence

<220>
<223> primer

<400> 25
ggtattgagg gtcgcatgcc agaattagct aatga   35

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<210>   26
<211>   37
<212>   DNA
<213>   Artificial sequence

<220>
<223>   primer

<400>   26
agaggagagt tagagcctta ttcgtcctct tgtaaaa                           37


<210>   27
<211>   35
<212>   DNA
<213>   Artificial sequence

<220>
<223>   primer

<400>   27
ggtattgagg gtcgcatgcg ctctacgacg gctcc                             35



<210>   28
<211>   37
<212>   DNA
<213>   Artificial sequence

<220>
<223>   primer

<400>   28
agaggagagt tagagcctca gccgcgcagc accttgg                           37


<210>   29
<211>   35
<212>   DNA
<213>   Artificial sequence

<220>
<223>   primer

<400>   29
ggtattgagg gtcgcatgtt tgagaacatt accgc                             35


<210>   30
<211>   37
<212>   DNA
<213>   Artificial sequence

<220>
<223>   primer

<400>   30
agaggagagt tagagcctta cagcactgcc acaatcg                           37
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<210>   31
<211>   1194
<212>   DNA
<213>   E. coli

<400>   31
gtgtttcaaa aagttgacgc ctacgctggc gacccgattc ttacgcttat ggagcgtttt      60

aaagaagacc ctcgcagcga caaagtgaat ttaagtatcg gtctgtacta caacgaagac     120

ggaattattc cacaactgca agccgtggcg gaggcggaag cgcgcctgaa tgcgcagcct     180

catggcgctt cgctttattt accgatggaa gggcttaact gctatcgcca tgccattgcg     240

ccgctgctgt ttggtgcgga ccatccggta ctgaaacaac agcgcgtagc aaccattcaa     300

acccttggcg ctccggggc attgaaagtg ggcgcggatt tcctgaaacg ctacttcccg     360

gaatcaggcg tctgggtcag cgatcctacc tgggaaaacc gcgtagcaat attcgccggg     420

gctggattcg aagtgagtac ttacccctgg tatgacgaag cgactaacgg cgtgcgcttt     480

aatgacctgt tggcgacgct gaaaacatta cctgcccgca gtattgtgtt gctgcatcca     540


tgttgccaca acccaacggg tgccgatctc actaatgatc agtgggatgc ggtgattgaa     600

attctcaaag cccgcgagct tattccattc ctcgatattg cctatcaagg atttggtgcc     660

ggtatggaag aggatgccta cgctattcgc gccattgcca gcgctggatt acccgctctg     720

gtgagcaatt cgttctcgaa aattttctcc ctttacggcg agcgcgtcgg cggactttct     780

gttatgtgtg aagatgccga agccgctggc cgcgtactgg ggcaattgaa agcaacagtt     840

cgccgcaact actccagccc gccgaatttt ggtgcgcagg tggtggctgc agtgctgaat     900

gacgaggcat tgaaagccag ctggctggcg gaagtagaag agatgcgtac tcgcattctg     960

gcaatgcgtc aggaattggt gaaggtatta agcacagaga tgccagaacg caatttcgat    1020

tatctgctta tcagcgcgg catgttcagt tataccggtt taagtgccgc tcaggttgac    1080

cgactacgtg aagaatttgg tgtctatctc atcgccagcg tcgcatgtg tgtcgccggg    1140

ttaaatacgg caaatgtaca acgtgtggca aaggcgtttg ctgcggtgat gtaa         1194


<210>   32
<211>   397
<212>   PRT
<213>   E. coli

<400>   32

Val Phe Gln Lys Val Asp Ala Tyr Ala Gly Asp Pro Ile Leu Thr Leu
1               5                   10                  15
```

```
Met Glu Arg Phe Lys Glu Asp Pro Arg Ser Asp Lys Val Asn Leu Ser
         20              25              30

Ile Gly Leu Tyr Tyr Asn Glu Asp Gly Ile Ile Pro Gln Leu Gln Ala
         35              40              45

Val Ala Glu Ala Glu Ala Arg Leu Asn Ala Gln Pro His Gly Ala Ser
    50              55              60

Leu Tyr Leu Pro Met Glu Gly Leu Asn Cys Tyr Arg His Ala Ile Ala
65              70              75              80

Pro Leu Leu Phe Gly Ala Asp His Pro Val Leu Lys Gln Gln Arg Val
             85              90              95

Ala Thr Ile Gln Thr Leu Gly Gly Ser Gly Ala Leu Lys Val Gly Ala
             100             105             110

Asp Phe Leu Lys Arg Tyr Phe Pro Glu Ser Gly Val Trp Val Ser Asp
         115             120             125

Pro Thr Trp Glu Asn Arg Val Ala Ile Phe Ala Gly Ala Gly Phe Glu
    130             135             140

Val Ser Thr Tyr Pro Trp Tyr Asp Glu Ala Thr Asn Gly Val Arg Phe
145             150             155             160

Asn Asp Leu Leu Ala Thr Leu Lys Thr Leu Pro Ala Arg Ser Ile Val
             165             170             175

Leu Leu His Pro Cys Cys His Asn Pro Thr Gly Ala Asp Leu Thr Asn
         180             185             190

Asp Gln Trp Asp Ala Val Ile Glu Ile Leu Lys Ala Arg Glu Leu Ile
         195             200             205

Pro Phe Leu Asp Ile Ala Tyr Gln Gly Phe Gly Ala Gly Met Glu Glu
    210             215             220

Asp Ala Tyr Ala Ile Arg Ala Ile Ala Ser Ala Gly Leu Pro Ala Leu
225             230             235             240

Val Ser Asn Ser Phe Ser Lys Ile Phe Ser Leu Tyr Gly Glu Arg Val
             245             250             255

Gly Gly Leu Ser Val Met Cys Glu Asp Ala Glu Ala Ala Gly Arg Val
```

```
                260                      265                      270
```

Leu Gly Gln Leu Lys Ala Thr Val Arg Arg Asn Tyr Ser Ser Pro Pro
    275   280   285

Asn Phe Gly Ala Gln Val Val Ala Ala Val Leu Asn Asp Glu Ala Leu
    290   295   300

Lys Ala Ser Trp Leu Ala Glu Val Glu Glu Met Arg Thr Arg Ile Leu
305   310   315   320

Ala Met Arg Gln Glu Leu Val Lys Val Leu Ser Thr Glu Met Pro Glu
                325   330   335

Arg Asn Phe Asp Tyr Leu Leu Asn Gln Arg Gly Met Phe Ser Tyr Thr
                340   345   350

Gly Leu Ser Ala Ala Gln Val Asp Arg Leu Arg Glu Glu Phe Gly Val
            355   360   365

Tyr Leu Ile Ala Ser Gly Arg Met Cys Val Ala Gly Leu Asn Thr Ala
        370   375   380

Asn Val Gln Arg Val Ala Lys Ala Phe Ala Ala Val Met
385   390   395

<210>   33
<211>   35
<212>   DNA
<213>   Artificial sequence

<220>
<223>   primer

<400>   33
ggtattgagg gtcgcgtgtt tcaaaaagtt gacgc   35

<210>   34
<211>   37
<212>   DNA
<213>   Artificial sequence

<220>
<223>   primer

<400>   34
agaggagagt tagagcctta catcaccgca gcaaacg   37

<210>   35
<211>   35

```
<212>  DNA
<213>  Artificial sequence

<220>
<223>  primer

<400>  35
ggtattgagg gtcgcatgga gtccaaagtc gttga                                    35


<210>  36
<211>  37
<212>  DNA
<213>  Artificial sequence

<220>
<223>  primer

<400>  36
agaggagagt tagagcctta cacttggaaa acagcct                                  37


<210>  37
<211>  35
<212>  DNA
<213>  Artificial sequence

<220>
<223>  primer

<400>  37
ggtattgagg gtcgcatgaa aaactggaaa acaag                                    35


<210>  38
<211>  37
<212>  DNA
<213>  Artificial sequence

<220>
<223>  primer

<400>  38
agaggagagt tagagcctta cagcttagcg ccttcta                                  37


<210>  39
<211>  35
<212>  DNA

<213>  Artificial sequence

<220>
<223>  primer

<400>  39
ggtattgagg gtcgcatgcg aggggcatta ttcaa                                    35


<210>  40
<211>  36
```

```
<212>  DNA
<213>  Artificial sequence

<220>
<223>  primer

<400>  40
agaggagagt tagagcctca gcccttgagc gcgaag                            36


<210>  41
<211>  1416
<212>  DNA
<213>  E. coli

<400>  41
atggaaaact ttaaacatct ccctgaaccg ttccgcattc gtgttattga gccagtaaaa    60

cgtaccaccc gcgcttatcg tgaagaggca attattaaat ccggtatgaa cccgttcctg   120

ctggatagcg aagatgtttt tatcgattta ctgaccgaca gcggcaccgg ggcggtgacg   180

cagagcatgc aggctgcgat gatgcgcggc gacgaagcct acagcggcag tcgtagctac   240

tatgcgttag ccgagtcagt gaaaaatatc tttggttatc aatacaccat tccgactcac   300

cagggccgtg gcgcagagca aatctatatt ccggtactga ttaaaaaacg cgagcaggaa   360

aaaggcctgg atcgcagcaa aatggtggcg ttctctaact atttctttga taccacgcag   420

ggccatagcc agatcaacgg ctgtaccgtg cgtaacgtct atatcaaaga agccttcgat   480

acgggcgtgc gttacgactt taaaggcaac tttgaccttg agggattaga acgcggtatt   540

gaagaagttg gtccgaataa cgtgccgtat atcgttgcaa ccatcaccag taactctgca   600

ggtggtcagc cggtttcact ggcaaactta aaagcgatgt acagcatcgc gaagaaatac   660

gatattccgg tggtaatgga ctccgcgcgc tttgctgaaa acgcctattt catcaagcag   720

cgtgaagcag aatacaaaga ctggaccatc gagcagatca cccgcgaaac ctacaaatat   780

gccgatatgc tggcgatgtc cgccaagaaa gatgcgatgg tgccgatggg cggcctgctg   840

tgcatgaaag acgacagctt ctttgatgtg tacaccgagt gcagaaccct ttgcgtggtg   900

caggaaggct ccccgacata tggcggcctg gaaggcggcg cgatggagcg tctggcggta   960

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ctggtcgatg gtctggaaga gattggcgtt gtctgccagc aggcgggcgg tcacgcggca  1080

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ctggcctgcg agctgtataa agtcgccggt atccgtgcgg tagaaattgg ctctttcctg  1200

ttaggccgcg atccgaaaac cggtaaacaa ctgccatgcc cggctgaact gctgcgttta  1260

accattccgc gcgcaacata tactcaaaca catatggact tcattattga agcctttaaa  1320

catgtgaaag agaacgcggc gaatattaaa ggattaacct ttacgtacga accgaaagta  1380
```

```
ttgcgtcact  tcaccgcaaa  acttaaagaa  gtttaa                              1416
```

```
<210>   42
<211>   1371
<212>   DNA
<213>   Citrobacter freundii

<400>   42
atgaattatc  cggcagaacc  cttccgtatt  aaaagcgttg  aaactgtatc  tatgatcccg      60

cgtgatgaac  gcctcaagaa  aatgcaggaa  gcgggttaca  atactttcct  gttaaattcg     120

aaagatattt  atattgacct  gctgacagac  agtggcacta  acgcaatgag  cgacaagcag     180

tgggccggaa  tgatgatggg  tgatgaagcg  tacgcgggca  gcgaaaactt  ctatcatctg     240

gaaagaaccg  tgcaggaact  gtttggcttt  aaacatattg  ttccgactca  ccagggggcgt     300

ggcgcagaaa  acctgttatc  gcagctggct  attaaacctg  gcaatatgt  tgccgggaat     360

atgtatttca  ctaccacccg  ttatcaccag  gaaaaaaatg  gtgcggtgtt  tgtcgatatc     420

gttcgtgacg  aagcgcacga  tgccggtctg  aatattgcgt  ttaaaggtga  tatcgatctt     480

aaaaaattac  aaaagctgat  tgatgaaaaa  ggcgcagaga  atattgcgta  tatctgcctg     540

gcggtgacgg  ttaacctcgc  gggcggccaa  ccggtctcga  tggctaacat  gcgtgcggtg     600

cgtgaactga  cagaagcgca  tggcattaaa  gtgttctacg  acgctacccg  ctgcgtagaa     660

aacgcctact  ttatcaaaga  gcaagagcag  ggctttgaga  caagagcat  cgccgagatc     720

gtgcatgaga  tgttcagcta  cgccgacggt  tgtaccatga  gtggtaaaaa  agactgtctg     780

gtgaacatcg  gcggcttcct  gtgcatgaac  gatgacgaaa  tgttctcttc  tgccaaagag     840

ttagtcgtgg  tctacgaagg  gatgccatct  tacggcggcc  tggccggacg  tgatatggaa     900

gcgatggcga  ttggcctgcg  tgaagccatg  cagtacgaat  atattgagca  ccgcgtgaag     960

caggttcgct  acctgggcga  taagctgaaa  gccgctggcg  taccgattgt  tgaaccggta    1020

ggcggtcacg  cggtattcct  cgatgcgcgt  cgcttctgcg  agcatctgac  gcaagatgag    1080

ttcccggcac  aaagtctggc  tgccagcatc  tatgtggaaa  ccggcgtgcg  cagtatggag    1140

cgcggaatta  tctctgcggg  ccgtaataac  gtgaccggtg  aacaccacag  accgaaactg    1200

gaaaccgtgc  gtctgactat  tccacgtcgt  gtttatacct  acgcacatat  ggatgttgtg    1260

gctgacggta  ttattaaact  ttaccagcac  aaagaagata  ttcgcgggct  gaagtttatt    1320

tacgagccga  agcagttgcg  tttctttact  gcacgctttg  attacatcta  a             1371
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<220>
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agaggagagt tagagcctca atcgatatat ttcagtc   37

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aagccctata tgcgccccat ctatgccggc aagcaggtct cgggcaccgc cgtcacggtg   180

ctgctgcagc ccggcgacaa ctggatgatg catgtggctg ccgagcagat tcagcccggc   240

gacatcgtgg tcgcagccgt caccgcagag tgcaccgacg gctacttcgg cgatctgctg   300

gccaccagct tccaggcgcg cggcgcacgt gcgctgatca tcgatgccgg cgtgcgcgac   360

gtgaagacgc tgcaggagat ggactttccg gtctggagca aggccatctc ttccaagggc   420

acgatcaagg ccaccctggg ctcggtcaac atccccatcg tctgcgccgg catgctggtc   480

acgcccggtg acgtgatcgt ggccgacgac gacggcgtgg tctgcgtgcc cgccgcgcgt   540

gccgtggaag tgctggccgc cgcccagaag cgtgaaagct cgaaggcga aaagcgcgcc   600

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Ala Ala Ala Asp Gly Leu Ala Ala Leu Gly Ser Ala Thr Val His Glu

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Ala  Met  Gly  Arg  Val  Gly  Leu  Leu  Lys  Pro  Tyr  Met  Arg  Pro  Ile  Tyr
          35                      40                      45

Ala  Gly  Lys  Gln  Val  Ser  Gly  Thr  Ala  Val  Thr  Val  Leu  Leu  Gln  Pro
          50                      55                      60

Gly  Asp  Asn  Trp  Met  Met  His  Val  Ala  Ala  Glu  Gln  Ile  Gln  Pro  Gly
65                       70                      75                      80

Asp  Ile  Val  Val  Ala  Ala  Val  Thr  Ala  Glu  Cys  Thr  Asp  Gly  Tyr  Phe
                    85                      90                      95

Gly  Asp  Leu  Leu  Ala  Thr  Ser  Phe  Gln  Ala  Arg  Gly  Ala  Arg  Ala  Leu
          100                     105                     110

Ile  Ile  Asp  Ala  Gly  Val  Arg  Asp  Val  Lys  Thr  Leu  Gln  Glu  Met  Asp
          115                     120                     125

Phe  Pro  Val  Trp  Ser  Lys  Ala  Ile  Ser  Ser  Lys  Gly  Thr  Ile  Lys  Ala
          130                     135                     140

Thr  Leu  Gly  Ser  Val  Asn  Ile  Pro  Ile  Val  Cys  Ala  Gly  Met  Leu  Val
145                      150                     155                     160

Thr  Pro  Gly  Asp  Val  Ile  Val  Ala  Asp  Asp  Gly  Val  Val  Cys  Val
                    165                     170                     175

Pro  Ala  Ala  Arg  Ala  Val  Glu  Val  Leu  Ala  Ala  Ala  Gln  Lys  Arg  Glu
               180                     185                     190

Ser  Phe  Glu  Gly  Glu  Lys  Arg  Ala  Lys  Leu  Ala  Ser  Gly  Ile  Leu  Gly
          195                     200                     205

Leu  Asp  Met  Tyr  Lys  Met  Arg  Glu  Pro  Leu  Glu  Lys  Ala  Gly  Leu  Lys
          210                     215                     220

Tyr  Ile  Asp
225


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<400>  68
cggctgtcga ccgttagtca atatatttca ggc                              33


<210>  69
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<400>  69
cgcggatcca taatggttga gaacattacc g                                31


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acgcgtcgac ttacagcact gccacaatcg                                  30


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ccggaattca taatggtcga actgggagtt gt                               32


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<210> 73
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<400> 73
ggtattgagg gtcgc   15

<210> 74
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<220>
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<400> 74
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